UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

February 12, 2010

GRADUATE PROGRAMS IN THE BIOMEDICAL SCIENCES AND PUBLIC HEALTH

Ph.D. Degrees

Interdisciplinary
-Emerging Infectious Diseases
-Molecular & Cell Biology
-Neuroscience

Departmental
-Clinical Psychology
-Environmental Health Sciences
-Medical Psychology
-Medical Zoology

Physician Scientist (MD/Ph.D.)

Doctor of Public Health (Dr.P.H.)

Master of Science Degrees

-Public Health

Masters Degrees

-Health Administration & Policy
-Military Medical History
-Public Health
-Tropical Medicine & Hygiene

Graduate Education Office

Eleanor S. Metcalf, Ph.D., Associate Dean
Bettina Arnett, Support Specialist
Roni Bull, Support Specialist
Katie Hall, Support Specialist

Web Site
http://www.usuhs.mil/graded/

E-mail Address
graduateprogram@usuhs.mil

Phone Numbers
Commercial: 301-295-9474 / 3913
Toll Free: 800-772-1747
DSN: 295-9474
FAX: 301-295-6772

DISSERTATION APPROVAL
FOR THE DOCTORAL DISSERTATION
IN THE MOLECULAR AND CELL BIOLOGY
GRADUATE PROGRAM

Title of Dissertation: "Mechanism of hepatocyte growth factor inhibition of angiotensin II-induced apoptosis in primary lung cells"

Name of Candidate: Young H. Lee
Doctor of Philosophy Degree
February 19, 2010

DISSERTATION AND ABSTRACT APPROVED:

DATE: 2/19/2010

Dr. Tao-Yiao John Wu
DEPARTMENT OF OBSTETRICS/GYNECOLOGY
Committee Chairperson

Feb 19, 2010

Dr. Regina M. Day
DEPARTMENT OF PHARMACOLOGY
Dissertation Advisor

2/19/2010

Dr. Joseph McCabe
DEPARTMENT OF ANATOMY, PHYSIOLOGY & GENETICS
Committee Member

2/19/2010

Dr. Xin Xiang
DEPARTMENT OF BIOCHEMISTRY
Committee Member

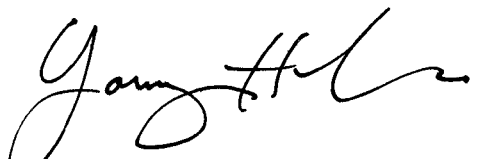
2-19-10

Dr. Michael Schell
DEPARTMENT OF PHARMACOLOGY
Committee Member

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

Mechanism of Hepatocyte Growth Factor Inhibition of Angiotensin II-induced Apoptosis in Primary Lung Cells

is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.



Young H. Lee
Molecular and Cell Biology, Dept of Pharmacology
Uniformed Services University
02/23/2010

Abstract

Title of Dissertation: "Mechanism of hepatocyte growth factor inhibition of angiotensin II-induced apoptosis in primary lung cells"

Author: Young H. Lee, Ph.D.
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road, Bethesda, MD 20814

Advisor: Regina M. Day, Ph.D.
Associate Professor
Department of Pharmacology
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road, Bethesda, MD 20814

Pulmonary fibrosis is a progressive disease resulting from the abnormal regeneration of cells after apoptosis. The air sacs in the lungs and their supporting structures become inflamed and scarred. As the scarring continues, the lungs stiffen due to the deposition of collagens and other extracellular matrix proteins. The thickening and stiffing of the lung eventually leads to difficulty breathing and lack of oxygen in the bloodstream. Currently, there are no treatments for pulmonary fibrosis, other than lung transplantation.

Angiotensin II (Ang II) is a key pro-apoptotic factor in fibrotic tissue diseases. However, the mechanism of Ang II-induced cell death in endothelial cells has not been previously elucidated. Here, I have identified a novel mechanism for Ang II-induced apoptosis that involves SHP-2-induced destabilization of Bcl-x_L mRNA through reduction of nucleolin binding. The degradation of Bcl-x_L mRNA caused an increase in the ratio of pro- to anti-apoptotic proteins, resulting in mitochondrial outer membrane permeabilization and subsequent cellular apoptosis.

Hepatocyte growth factor (HGF), a potent endogenous tissue repair factor, has been demonstrated to attenuate lung fibrosis in murine models, but the mechanism is yet unknown. I hypothesized that HGF may prevent endothelial cell apoptosis induced by Ang II. Here, I demonstrated the ability of HGF to inhibit Ang II-induced apoptosis by stabilizing the Bcl-x_L mRNA through the phosphorylation and cytoplasmic localization of the nucleolin in both *in vitro* and in *ex vivo* lung tissue explants. The known mechanism of HGF inhibition of Ang II-induced apoptosis may provide novel targets for the mitigation of fibrotic diseases in future.

Title Page

Mechanism of hepatocyte growth factor inhibition of angiotensin II-induced apoptosis in primary lung cells

By

Young Hwan Lee

Dissertation submitted to the Faculty of the Molecular and Cell Biology Program of the Uniformed Services University of the Health Sciences in partial fulfillments for the degree of

Doctor of Philosophy, 2010

Preface

Stand still and consider the wondrous works of God.

Job 37:14

Dedication

I dedicate this thesis to my mom and dad, who sacrificed so much so that I could have every opportunity to succeed. This is also for my sister who wanted me to achieve this goal as much as I did. Your overflowing encouragements, lifetime of love and support are the foundation of this thesis. I could not have done this without any of you. Kam sa hab ne da. Sa rang heh yo.

Acknowledgements

This project would not have been possible without the support, enthusiasm, encouragement, and creative insight of many special people over the years.

I am very grateful to the members of my dissertation committee for their guidance and encouragement throughout my tenure as a doctoral candidate: Dr. Regina M. Day, Dr. Tao-Yiao John Wu, Dr. Joseph McCabe, Dr. Michael Schell, and Dr. Xin Xiang. I consider myself to be extremely privileged to have had so many great individuals commit to the development of me as a scientist. Thank you.

I am most grateful to my thesis advisor, Dr. Regina M. Day, for her unwavering support, insights, patience…and of course, her zucchini chocolate cake! Gina, thank you for helping me grow as a scientist. You are an amazing role model and you have changed my life in countless positive ways, and I am deeply grateful. Working with you has been an intellectually stimulating and wonderful experience, both on a professional and personal level.

I want to thank Dr. Yuichiro J. Suzuki, who allowed me to come into his lab with very limited science experience and attempting to teach me how to become a scientist. Thank you, Justin, for your guidance, advices, witty stories, and great parties. I would not be where I am today if you did not take a chance on me.

From my first day of school to today, and from the formative stages of this thesis to the final draft, I owe an immense debt of gratitude to Dr. Randall Merling and Dr. Bethanie Morrison. Thanks, Randall. I will see you over at the tennis court later. I am especially grateful to Bethanie Morrison, who stood beside me and urged me at every step of the way. Beth, thank you for everything, especially your encouragements and support that provided me with confidence and your witty dialogue that always made me smile. From helping me edit the thesis to trying to find the best coffee in town (…still looking), I could not have it done it without your help.

I am very grateful to the members of Day lab, past and present, who have spent countless hours advising and helping me. Their mentorship and friendship made the long hours spent in the lab enjoyable. Thank you to Dr. Mungunsukh Ognoon, Ronald Panganiban, Ana Marquez, Elizabeth McCart, and Autumn Griffin.

The list of friends is too long to write, but I will always remember the wonderful friendship I shared with my fellow students. And I am grateful for the wonderful interaction I had had with the faculty of the Molecular and Cell Biology Program and the Pharmacology Department. Thank you all for the great memories.

Table of Contents

Contents

Table of Figures

Chapter 1

Objectives

Idiopathic pulmonary fibrosis (IPF) is a disease that is steadily increasing in frequency without any discrepancies in race, age, sex, or socio-economic distributions. Currently, the pathophysiology of this disease is not clearly understood, and there is no curative therapy short of organ transplant. Since most patients are diagnosed with the disease in a late and often fatal stage, organ transplant may not be an option, thus new therapeutic strategies are desperately needed. Pulmonary fibrosis is characterized by the progressive loss of normal lung architecture associated with the apoptosis of alveolar epithelial and endothelial cells, which leads to the proliferation of activated fibroblasts and alterations in the extracellular matrix. Activated fibroblasts or myofibroblasts are believed to play a central role in the development and progression of the disease.

While angiotensin II (Ang II) has predominately been studied for its role in blood pressure homeostasis, it has also been strongly correlated to the development and progression of pulmonary fibrosis. In both *in vitro* and *in vivo* studies, Ang II mediates growth and transdifferentiation of myofibroblasts. Ang II also has been shown to induce epithelial and endothelial cell apoptosis. Human patients with pulmonary fibrosis expressed increased level of Ang II and angiotensin converting enzyme. While the effect of Ang II has been linked to pulmonary fibrosis disease, the signaling mechanism of Ang II-induced endothelial apoptosis is still unknown.

Antithetical to Ang II, hepatocyte growth factor (HGF), an endogenous growth factor, contributes to epithelial and endothelial cell survival. Animal studies show that administration of HGF induces proliferation of epithelial and endothelial cells, promotes normal tissue regeneration, and inhibits the development of fibrosis. The

simultaneous or delayed administration of HGF to mice undergoing bleomycin-induced lung injury inhibits both endothelial and epithelial cell apoptosis, the manifestation of fibroblast foci, and the deposition of collagen typically found in pulmonary fibrosis. Overall, HGF appears to have the ability to mediate tissue repair in a manner that evades aberrant tissue remodeling pathways, yet the mechanism remains unknown. Based on these findings, I **hypothesize that fibrosis in lung injury and IPF is mediated by Ang II**. I further hypothesize that **HGF acts as a major effector, involved in the repair and protection of the lung by interfering with the downstream signal transduction cascade of Ang II.** Successful completion of the proposed research may lead to the development of a **targeted therapy for IPF that may successfully delay or prevent development of fibrosis.**

The main **objectives** of this research were to advance understanding with regard to the involvement of Ang II in IPF using a cell model system, and to develop the rationale for the targeted therapy of HGF in repressing or preventing fibrosis in the lung. To address these objectives, I proposed the following **specific aims**:

1) Identify signaling mechanisms required for Ang II-mediated apoptosis in the pulmonary artery endothelial cells
2) Determine the anti-apoptotic mechanisms of HGF in Ang II-induced apoptosis in the pulmonary artery endothelial cells

The **long term goal** of this study was to develop targeted therapy using HGF in IPF, with the principles that this molecule can be considered a biological response

modifier to prevent and also to repress fibrosis in lung injuries by targeting relevant biomarkers.

Chapter 2

Significance

Data from human studies and animal models indicate that apoptosis of pulmonary epithelial and endothelial cells is a critical event in acute lung injury, adult respiratory distress syndrome (ARDS), and may be a causative factor leading to the development of pulmonary fibrosis [1]. Increased levels of apoptotic factors have been detected in bronchoalveolar lavage fluid from ARDS patients, acute lung injury, and pulmonary fibrosis [2-5]. Apoptosis has been documented in bronchial and alveolar epithelial cells adjacent to fibrotic plaques from patients with idiopathic and bleomycin-induced pulmonary fibrosis [6-9]. Epithelial and endothelial cell apoptosis is a key feature in animal models of lung injury and fibrosis [10-13]. Bleomycin, a chemotherapeutic agent which is commonly used as an inducer of pulmonary fibrosis in the animal model, produces apoptosis in bronchial and alveolar epithelial cells during the progression of the disease [14]. Furthermore, induction of apoptosis alone is sufficient to induce pulmonary fibrosis [15]; for example, an antibody against the death receptor-activating protein Fas was aerosolized into the mice and induced alveolar epithelial apoptosis and ultimately resulted in pulmonary fibrosis after inhalation [15]. Importantly, the inhibition of cellular apoptosis using a caspase inhibitor reduced the number of apoptotic cells as well as collagen deposition, and stopped the progression of fibrosis in mice treated with bleomycin [16-18]. Overall, human and animal data indicate that epithelial and endothelial cell apoptosis plays a significant role in acute lung injury, ARDS, and the development of pulmonary fibrosis. By understanding the mechanism of cellular apoptosis in the pulmonary fibrosis, therapeutic options can be designed and help prevent the progression of the disease.

Chapter 3

Introduction

Physiology of the lung

The primary function of the lung is to provide a highly efficient area for rapid gas exchange, which is achieved by a well synchronized interaction of the lungs with the central nervous system, circulatory system, chest wall musculature, and the diaphragm. In addition, the lung serves as a reservoir for blood and to move air to and from the exchange surfaces of the lungs. The lung also protects the respiratory epithelia from dehydration, temperature changes, and invasion by pathogens. Respiratory diseases interfere with ventilation, blood flow, and gas exchange, which will ultimately lead to respiratory failure and death.

There are two primary functions for which the lung is structured: 1) to transport air to and from the lung and nose/mouth (ventilation) and 2) to allow gas exchange from the lung to the rest of the body (circulatory system for perfusion). The respiratory tract consists of the airways which carry air to and from the exchange surfaces of the lungs. At the distal end, the trachea divides into a pair of respiratory branches. The conducting airways consist of about 23 divisions of branching tubes including main bronchi, lobar bronchi, segmental bronchi, leading to terminal bronchioles that become smaller and shorter as they infiltrate deeper into the lung. The main function of the lung is to achieve inspired gas to the gas-exchange regions of the lung. Gas exchange takes place in the alveolus, where the laminar blood flow and the inhaled air are divided by a thin layer of tissue (Figure 1). Only ~10% of the normal healthy lung is occupied by solid tissue; the majority of the lung is filled with

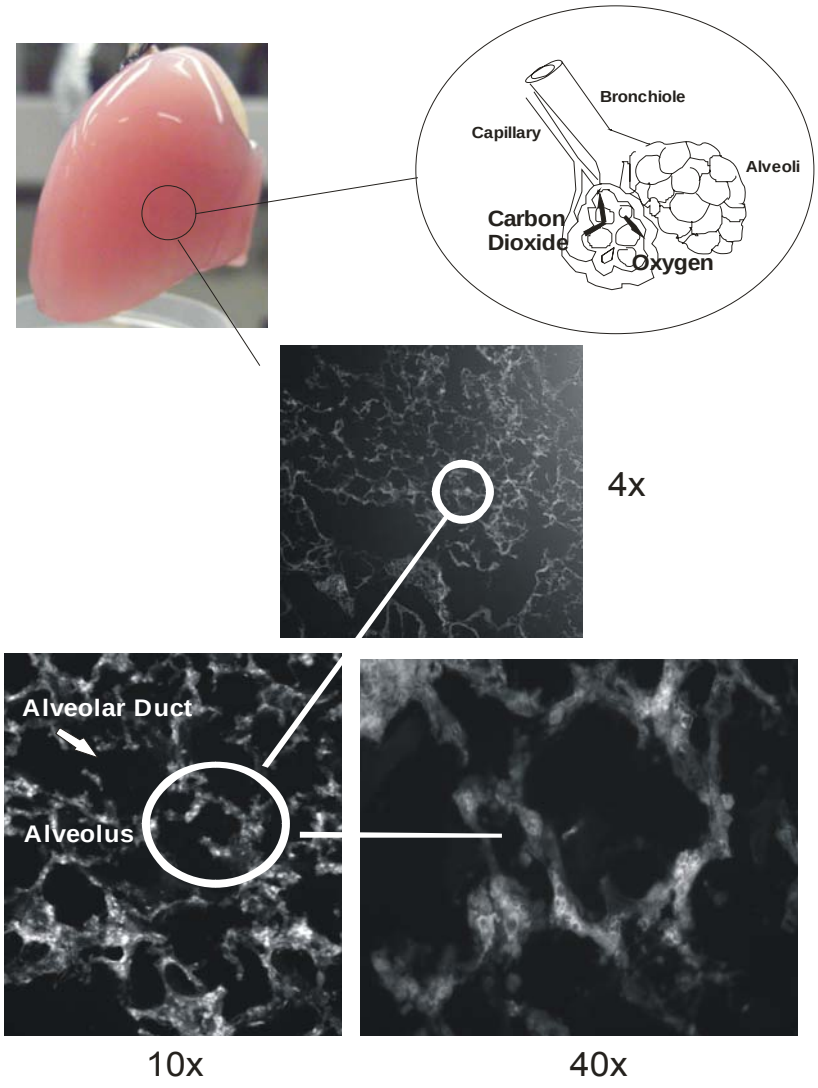


Figure 1: Physiology of the Lung. When the air enters through the nose or mouth, it travels through the trachea into the chest. The trachea branches into two bronchi, one going to each lung. Inside the lungs, the bronchi branches into smaller bronchi and then into bronchioles. Each bronchiole has air sacs or alveoli at its end. The blood flows between the thin walls of nearby alveoli, allowing oxygen to move from the air into the blood and carbon dioxide to move from the blood to the air, to be breathed out.

air and blood. While the lung structure is delicate as to allow gas exchange, it must also be sturdy to maintain the architectural integrity to sustain the alveolar structure.

Although the lung has no function during the fetal stage, only to assume a vital role starting at the birth, by the late stage of gestation the lung acquires all of the essential components for full morphological development [19]. Fundamental to this development are the connective tissue elements, which play an important role in lung morphology and mechanical properties [19]. The lung has two well defined interstitial connective tissue compartments, the connective tissue (e.g., peribronchovascular sheaths, interlobular septa, and visceral pleura) and the parenchymal interstitium [20]. These two compartments have anatomical and functional differences. Collagen type IV is mainly located in the parenchymal compartment where the basement membranes of the capillary endothelium and alveolar epithelium are found. The lymphatics are found mainly in the loose-binding connective tissue. The lung interstitium is predominantely occupied by a matrix composed of glycosaminoglycans [20], various interstitial cells such as mast cells, plasma cells and leukocytes, and fibers such as collagen types I and III, elastin, proteoglycans, fibronectin and reticulin [21-24]. Of these, collagen has the greatest tensile strength and plays a role in limiting lung expansion [25]. In the lung, collagen is found associated with blood vessels, bronchi, and the alveolar interstitium [26, 27].

The major physiological role of the lung is gas exchange, where the incoming fresh air is passed through the branching airways to the terminal respiratory units, where the mixing of gas occurs largely through molecular diffusion [28]. Capillaries bring blood with low levels of oxygen and high levels of carbon dioxide to the lung.

The carbon dioxide disseminates into the alveoli for removal from the bloodstream and oxygen is transferred to the capillaries; afterward, the oxygenated blood flows through pulmonary veins to the left heart for distribution to the tissues of the body [20].

Interstitial lung diseases

Under physiological conditions, the interstitial lung space is a fine and virtually invisible space between the basement membrane of the alveolar epithelium and the alveolar capillary endothelium (Figure 2)[29]. Small numbers of interstitial macrophages, fibroblasts and myofibroblasts are present. The extracellular matrix (ECM) proteins, primarily collagen and related macromolecules, as well as noncollagenous proteins such as fibronectin and laminin, are also present in the lung interstitium [30].

Deregulated production and degradation of the ECM in tissue remodeling after lung injury may lead to the amassing of ECM in the alveolar walls and spaces during pulmonary fibrosis. Various cells of the lung, such as macrophages, endothelial cells, alveolar and bronchiolar epithelial cells, and fibroblasts can produce ECM components following lung injury. The activated fibroblasts is one of the causes of exaggerated ECM production in fibrosing lung disease due to their ability to produce collagen [31]. Previous studies have demonstrated that lung injury induces inflammation either followed by or concurrently with the tissue remodeling process.

ECM proteins have many biological functions, including the modulation of inflammatory and fibrotic processes. The ECM has an important role in enhancing the

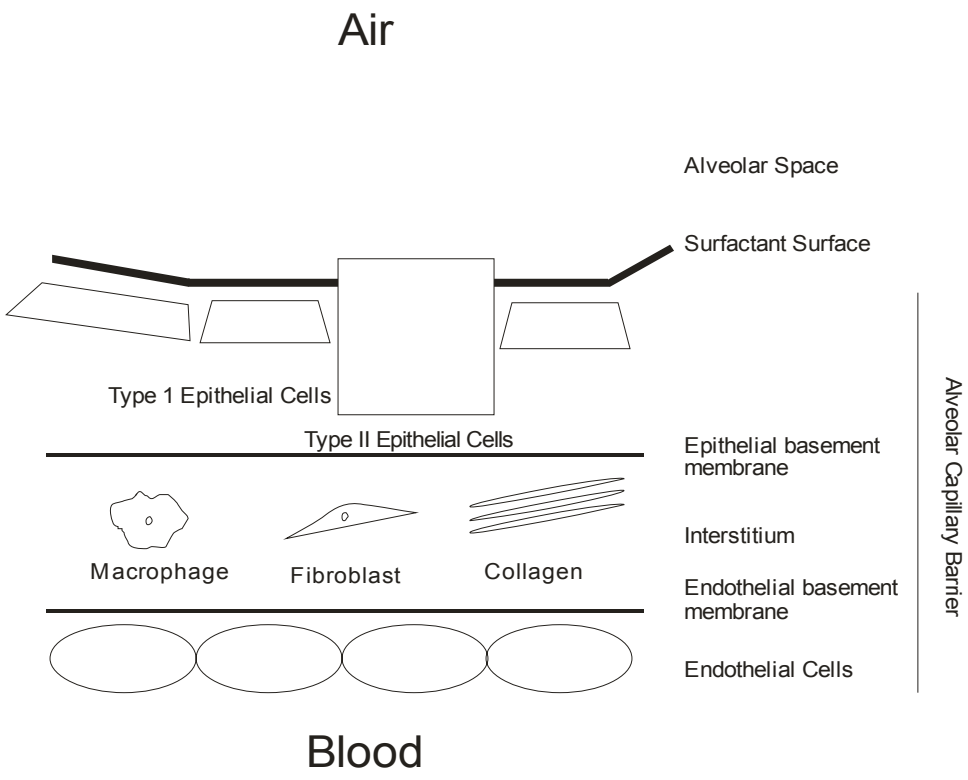


Figure 2: The diagram of the main constituents of alveolus. The epithelium of the alveoli contains two types of cells. 1) Type 1 pneumocytes or large flattened cells. They cover 95% of the total alveolar area, which present a thin diffusion barrier for gases. They are connected to each other by tight junctions. 2) Type 2 pneumocytes amke up 5% of the total alveolar area but is 60% of total number of cells. Type 2 epithelial cells secrete surfactant, which decreases the surface tension betw een the alveolar walls. These cells are connected by tight junctions. The intersititial space, interposed between the endothelial and epithelial cells, is composed of three dimensional scaffold of insoluble macromolecules around the interstitial cells composed mostly of fibroblasts and macrophages; the space is also filled with a solution of soluble solutes of various molecular weight, from salts to large molecular weight plasma protein, dispersed in a aquous environment. Endothelial cells of blood capillaries are continuous and non-fenestrated. The thin layer of endothelial cells allow a place for gas-exchange.

adhesion and migration of both inflammatory and non-inflammatory cells [32]. In addition, the ECM is chemotactic to various types of cells, and activates immune cells to yield inflammatory factors such as prostaglandins, superoxide and cytokines that enhance inflammation and fibrosis [33, 34]. ECM- derived signals may regulate cell proliferation and differentiation, as well as apoptosis [35].

Clinical and histological features of IPF

Idiopathic pulmonary fibrosis (IPF) is a severe and progressive disease [36]. The development of IPF involves gradual replacement of the lung tissue with fibrosis or scarring, typically resulting from microscopic injury to the lung [37]. In the past, the predominant theory was that the process of pulmonary fibrosis began with inflammation, which resulted in scar formation [38, 39]. However, it has recently been suggested that fibrosis itself, signfying abnormal wound repair, is the primary process while inflammation is secondary [38, 39].

Pulmonary fibrosis initiates in the tissue of the interstitium, which supports the structures of the lung, specifically alveoli [29]. The destruction of the alveolar architecture results in irreversible loss of pulmonary function and impaired gas exchange [40]; death usually occurs as the result of respiratory insufficiency [41]. While there are many lung diseases that lead to shortness of breath (dyspnea), they in general fall into two main gropus: obstructive disease or restrictive disease [42]. Obstructive lung diseases create airflow problems. Air can infiltrate the lung, but is trapped and has difficulty being released. Things that regulate or hamper the flow of

air include constraint or contraction of the breathing tubes as well as increased secretions and swelling of the lining as a result of inflammation. Cystic fibrosis, asthma, bronchitis, emphysema, and chronic obstructive pulmonary disease are types of obstructive diseases. In contrast, pulmonary fibrosis and various pneumonias are types of restrictive diseases. Restrictive disease is a low-air-volume disorder, where the bloodstream cannot get enough oxygen due to thickened walls of the alveoli. In the case of pulmonary fibrosis, the disease primarily involves scarring of the lung, in which the air sacs of the lungs progressively become replaced with the fibrotic tissue. When the scar forms in the lung, the tissue becomes thicker resulting in an irreparable loss of the capacity of the tissue to transfer oxygen into the bloodstream.

Many acute and chronic lung disorders with varying levels of pulmonary inflammation and fibrosis are together characterized as interstitial lung diseases. Idiopathic pulmonary fibrosis (IPF) is defined as one of several forms of chronic fibrosing interstitial pneumonias limited to the lung and linked with the histologic appearance of interstitial pneumonia on surgical lung biopsy. The histology of IPF is the heterogenous appearance of alternating areas of normal lung with interstitial inflammation and fibrosis in the early stages, with extensive honeycombing and septal thickening in the later disease stages [43]. The pathology of pulmonary fibrosis in the lung is characterized by the apoptosis of epithelial and endothelial cells, proliferation of activated fibroblasts (myofibroblasts), and extensive deposition of collagen and other ECM proteins [44].

Initial Trigger of IPF

Fibrosis or scarring can sometimes be associated to particular causes such as prolonged exposure to occupational or environmental contaminants or dusts. These contaminants can include inorganic dusts such as talc, asbestos, silica, beryllium and hard metal dusts or organic dusts such as bacteria and animal proteins. Some related diseases include asbestosis and silicosis. Other potential risk factors identified for IPF, without a clear or significant connection, are cigarette smoking [45-47], exposure to prescribed drugs [48], and various other environmental factors [45, 46, 49-52]. IPF has also been connected with a number of infectious agents such as Epstein-Barr virus [53-55], hepatitis C [56-58], influenza [59-61] and cytomegalovirus [62]. Parainfluenza 1 virus [63], human immunodeficiency virus 1 [64], measles virus [65], parainfluenza 3 virus [66], herpesvirus 6 [67], *Mycoplasma* [68], and legionnaires' disease [69] have also been implicated as possible participants in the pathogenesis of IPF. Genetic predisposition to IPF has been taken into consideration [70-72] but no specific genetic markers have been identified [73].

Angiotensin II-mediated IPF

Recent studies strongly correlate angiotensin II (Ang II) with the development and progression of pulmonary fibrosis, primarily by inducing the growth and transdifferentiation of normal fibroblasts to myofibroblasts, as well as the induction of apoptosis in epithelial and endothelial cells [74]. Human lung myofibroblasts from patients with idiopathic pulmonary fibrosis were found to generate Ang II [75]. The

cellular actions of Ang II have been connected to the autocrine release of growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor β (TGFβ), which are all potent inducers of the myofibroblast activation and procollagen synthesis [76, 77]. **While the effect of Ang II on fibroblasts is an intensive and ongoing area of research, the signal transduction mechanism of Ang II leading to endothelial cell apoptosis remains unknown.**

Diagnosis of IPF

IPF progresses in an either harsh or insidious manner that may be difficult to expose using factors such as symptomatology, chest radiographic findings, or spirometry alone [78], thus the accurate incidence and frequency of IPF are not known. The course of pulmonary fibrosis varies from patient to patient. For some, the disease may advance slowly and gradually over several years, while for others it may develop rapidly. While some patients may notice symptoms ranging from moderate to severe, other patients stabilize for a period of time. Symptoms are not always present at the onset of the disease. Scarring may occur long before any symptoms develop. The main symptom of pulmonary fibrosis is dyspnea, or shortness of breath. Most patients often ignore the occasional difficulty with breathing, attributing it to old age or being physically unfit [41]. As the condition deteriorates and the damage to the lung becomes severe, breathlessness may occur with slight physical actions. Another indication of progressive disease includes a dry hacking

cough. Patients may notice flu-like symptoms such as fatigue, weight loss and aching muscles and joints. In addition, they may experience enlargement and bulb-like development of the fingertips and nails, a condition called clubbing [41]. Clinical deterioration of the patient's health is most frequently the result of disease progression. Respiratory failure is the main cause of mortality, accounting for about 40% of the deaths of patients with IPF [41]. Other causes of mortality in IPF patients include heart failure, ischemic heart disease, infection, and pulmonary emboli [41]. Currently, the incidence of IPF is estimated at 10.7 cases per 100,000 males and 7.4 cases per 100,000 females per year [79]. The average age at disease onset is 40-60 years but the disease can occur at any age. Although not common, IPF does occur in children as young as 3 years of age. Rarely, interstitial lung disease has been diagnosed in children less than one year of age. Life expectancy after diagnosis varies between 2 to 5 years with a 5-year survival of only 30% [80-87] .

Current Treatment for IPF

Many treatments of IPF are based on the concept that inflammation leads to pulmonary injury and fibrosis [88]. Initially, it was thought that inflammatory and immune effector cells accumulated within the pulmonary parenchyma, and as this alveolar and interstitial reaction continued, alveolar wall, vascular, and airway damage would continue [87]. Based on this theory, reparative processes would become inadequate or impaired due to the inflammatory response, thus resulting in fibrosis.

As a result, the lung parenchyma would be irreversibly disturbed and gas exchange function impaired.

While this early conceptualization of the pathogenesis of pulmonary fibrosis suggests several theoretical points of therapeutic intervention, the most common treatments have been anti-inflammatory medication and lung transplantation. Optimal therapy for IPF remains controversial [89]. Thus far, treatment plans have been based on eradicating or repressing the inflammatory component. **There are no pharmacological therapies that have been confirmed to alter or reverse the inflammatory process of IPF.** Conventional treatment options include corticosteroids [80, 90-93], immunosuppressive/cytotoxic agents (e.g. azathioprine, cyclophosphamide) [90, 94, 95], and antifibrotic agents targeting collagen depositions (e.g. colchicines or D-penicillamine) [72, 90, 96, 97] alone or in combination.

Pathogenesis of IPF

The pathogenesis of IPF is a controversial topic. Two main hypotheses have arisen in past years: an inflammatory model of IPF pathogenesis and epithelial/endothelial fibroblastic model.

Based on the inflammatory model, a chronic and unresolved inflammatory process is followed by an unrecognized insult which injures the lung and initiates lung fibrogenesis [38]. This theory is based on the idea that injury and inflammation of the alveolar-capillary constituents and the basement membrane lead to the loss of type I epithelial and endothelial cells. This would cause in the loss of alveolar space

integrity, proliferation and recruitment of stromal cells and the accumulation of ECM proteins [98]. However, there is little evidence that inflammation initiates and plays an important part in the disease. IPF patients taking anti-inflammatory drugs have shown no improvement or regression of fibrosis [43].

The second hypothesis is the epithelial/endothelial fibroblastic model, where the injury on the epithelial and endothelial cells causes the formation of fibroblastic foci, resulting in a deposition of ECM and abnormal lung structure [38]. The primary site of the injury and repair are these fibroblastic foci, which are regions of fibroblastic proliferation. Active proliferation and secretion of fibroblasts/myofibroblasts with exaggerated deposition of ECM leads to the fibrosis. The alveolar epithelial and endothelial cells are injured and undergo apoptosis, leading to abnormal cell regeneration. Numerous *in vitro* and *in vivo* studies demonstrate that altered fibroblasts during IPF fibrotic lung injury release angiotensin II and other soluble factors that are capable of inducing apoptosis and induce alveolar cell death [75, 99]. Cellular apoptosis then results in extensive deposition of ECM that causes the destruction of alveolar-capillary unit and loss of lung function [38, 43, 100-102].

Fibroblasts

Fibroblasts are the primary stromal cells that participate in repair and regenerative processes in human tissue and organs. The main function of the fibroblasts is to secrete ECM proteins to provide a tissue scaffold for normal repair

events after an injury. After the tissue repair, it is crucial for the fibroblasts and myofibroblasts to undergo apoptosis to return to the normal tissue architecture [103].

Fibroblasts are present in the adventitia of the vascular structures and airways of the normal human adult lung. The lung is comprised ~40% of the interstitial fibroblasts. They are usually cultured as adherent cells with spindle-shape morphology. The fibroblasts express interstitial collagens of type I and III [104].

In the normal lung, fibroblasts synthesize a small amount of matrix; however, activated myofibroblasts are considered the key effector cells responsible for the increased ECM deposition in IPF [38, 101]. Myofibroblasts are a differentiated fibroblast cell type that is characterized by elevated production of ECM [105]. The morphological change associated with the progression of fibrosis is the fibroblastic foci, which is characterized by a cluster of fibroblasts/myofibroblasts within the alveolar wall; it represents a site of acute lung injury, in which the alveolar cells are destroyed and the epithelial basement membrane is uncovered [37, 106-109]. The origin of fibroblast foci are unknown but possibilities include transdifferentiation of epithelial cells to fibroblast phenotypes during the process called epithelial-to-mesenchymal transition [110-112], recruitment of circulating fibroblast precursors called fibrocytes [113-115], and differentitation of resident fibroblasts [104].

In an attempt to repair the damaged area of the tissue, the newly proliferated fibroblasts move to the injured area where they transform into the myofibroblasts that contract the wound and assemble new ECM [107]. Afterward, programmed cell death (apoptosis) occurs to mediate the downregulation of myofibroblast while the epithelial and endothelial cells regenerate [101]. In IPF, there is increased and continuous

events after an injury. After the tissue repair, it is crucial for the fibroblasts and myofibroblasts to undergo apoptosis to return to the normal tissue architecture [103].

Fibroblasts are present in the adventitia of the vascular structures and airways of the normal human adult lung. The lung is comprised ~40% of the interstitial fibroblasts. They are usually cultured as adherent cells with spindle-shape morphology. The fibroblasts express interstitial collagens of type I and III [104].

In the normal lung, fibroblasts synthesize a small amount of matrix; however, activated myofibroblasts are considered the key effector cells responsible for the increased ECM deposition in IPF [38, 101]. Myofibroblasts are a differentiated fibroblast cell type that is characterized by elevated production of ECM [105]. The morphological change associated with the progression of fibrosis is the fibroblastic foci, which is characterized by a cluster of fibroblasts/myofibroblasts within the alveolar wall; it represents a site of acute lung injury, in which the alveolar cells are destroyed and the epithelial basement membrane is uncovered [37, 106-109]. The origin of fibroblast foci are unknown but possibilities include transdifferentiation of epithelial cells to fibroblast phenotypes during the process called epithelial-to-mesenchymal transition [110-112], recruitment of circulating fibroblast precursors called fibrocytes [113-115], and differentitation of resident fibroblasts [104].

In an attempt to repair the damaged area of the tissue, the newly proliferated fibroblasts move to the injured area where they transform into the myofibroblasts that contract the wound and assemble new ECM [107]. Afterward, programmed cell death (apoptosis) occurs to mediate the downregulation of myofibroblast while the epithelial and endothelial cells regenerate [101]. In IPF, there is increased and continuous

destroyed cellular contents are released into the cell surroundings, causing a strong inflammatory response and damage to surrounding cells [122].

The molecular mechanisms of the apoptotic signaling pathway, including the initiation, mediation, execution and the regulation, have been intensively explored. In mammals, various external signals can trigger two major apoptotic pathways, namely the death receptor-dependent extrinsic pathway or the mitochondrial-dependent intrinsic pathway.

1. Extrinsic apoptosis: In the extrinsic pathway, extracellular apoptotic signals, such as death ligands, bind to cell surface death receptors (Figure 3). Examples of these receptors include the tumor necrosis factor (TNF) receptor, TRAIL, or Fas receptor [123]. The stimulation of death receptors results in their oligomerization and subsequent recruitment of the Fas-associated death domain (FADD) and initiator pro-caspase-8, resulting in formation of the death inducing signaling complex (DISC). Caspase 8 activation is prior to the activation of downstream effector caspases of the cell death program, including caspase 3, 6, and 7 [124].

2. Intrinsic apoptosis: Intrinsic apoptosis is activated following intracellular signals including growth factor deprivation, oxidative stress, or DNA damage induced by chemicals or irradiation (Figure 3). Intrinsic signals initiate apoptosis via mitochondria dysregulation [125]. In this pathway, the mitochondria release cytochrome *c* to the cytoplasm to initiate a caspase cascade. Cytosolic cytochrome *c*

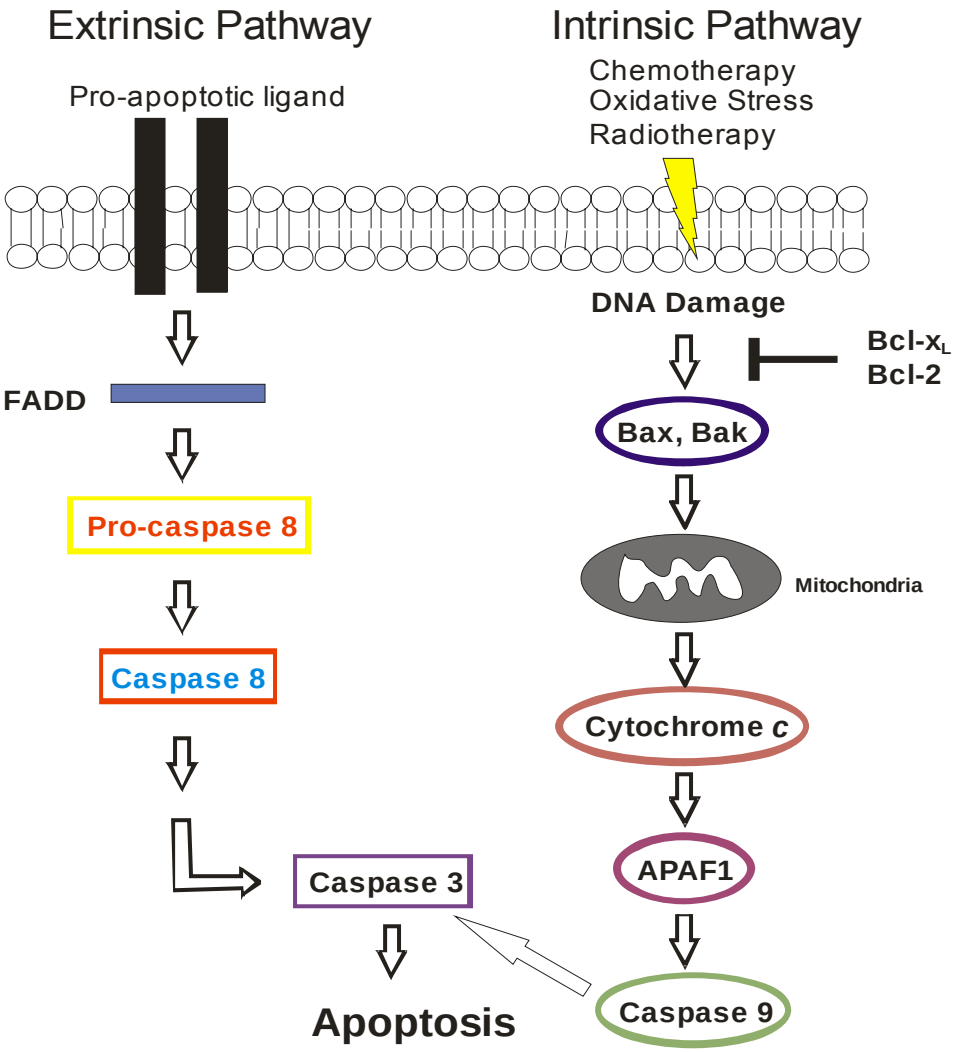


Figure 3: Extrinsic vs Intrinsic Pathway. The extrinsic pathway begins outside the cell through the binding and activation of death receptors on the cell surface by pro-apoptotic ligands. Extrinsic pathway requires activation of initiator caspase 8 to activate effector caspase 3 and apoptosis. The intrinsic apoptotic pathway hinges on the ratio between pro- and anti-apoptotic members of the Bcl-2 family, which act to regulate the permeability of the mitochondrial membrane and its release of cytochrome *c*. Initiator caspase 9 is activated in the intrinsic pathway, which cleaves and activates caspase 3 to induce apoptosis.

binds to apoptosis protease activator factor 1 (Apaf-1) and initiator pro-caspase 9. This forms an intracellular DISC-like complex called the apoptosome. Within the apoptosome, caspase 9 is activated, leading to the cleavage and activation of caspase 3 [126].

Cellular apoptosis is controlled by a complex network of pro- and anti-apoptotic molecules. Expression of anti-apoptotic molecules keeps the activation of pro-apoptotic factors in check [127]. Moreover, when apoptotic signals arise in the cells, pro-apoptotic factors can offset those inhibitory signals, resulting in a decreased ratio of anti-apoptotic to pro-apoptotic. The decrease in the ratio is sufficient to induce apoptosis (Figure 4) [128].

Mitochondrial outer membrane permeabilization (MOMP) is a key event of the intrinsic apoptotic process. MOMP is primarily regulated by Bcl-2 family members. Once MOMP occurs, cell death occurs through either the release of apoptotic molecules or by the loss of mitochondrial function that is essential for cell survival [129].

The approximately 20 Bcl-2 family members can be divided into three groups. Each group contains at least one of four conserved Bcl-2 Homology (BH) domains (Figure 5). Anti-apoptotic members such as Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1 promote cell survival. The other two groups promote cell death. The multi-BH domain proteins Bax, Bak, and Bok share three domains, BH1, BH2, and BH3, with Bcl-2 [121, 130]. BH3 proteins, such as Bid, Bad, and Bim possess only the short BH3 interaction domain.

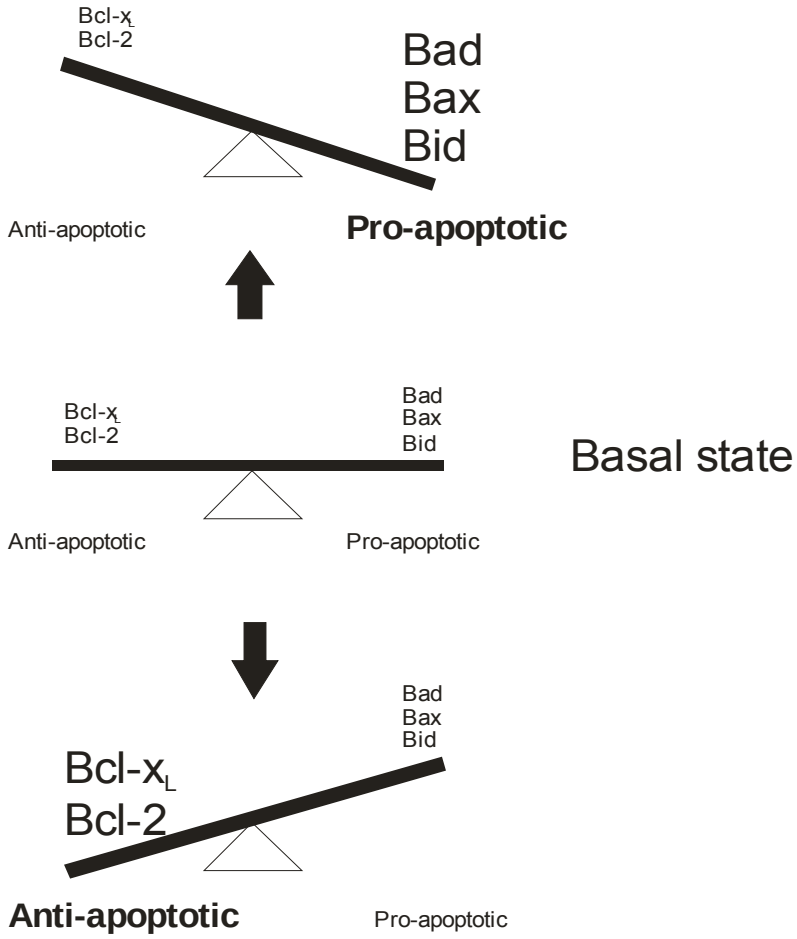


Figure 4: Balance between pro- and anti-apoptotic protein. The Bcl-2 family can be separated into three groups: anti-apoptotic members include Bcl-2, Bcl-x , Mcl-1, A1 and Bcl-w; pro-apoptotic proteins include Bax, Bak, and Bok; the BH3-only proteins include Bad, Bik Bid, Puma, Bim, Bmf, Noxa and Hrk. The interaction between the anti-and pro-apoptotic proteins led to a rheostat model which the ratio of pro-apoptotic and anti-apoptotic proteins controls cell fate. When the anti-apoptotic protein s bind to the pro-apoptotic, it neutralizes the death-inducing effects. Increase in the pro-apoptotic proteins lead to higher rate of pro-apoptotic dimerization and subsequent apoptosis. Increase in the anti-apoptotic proteins signify cell survival.

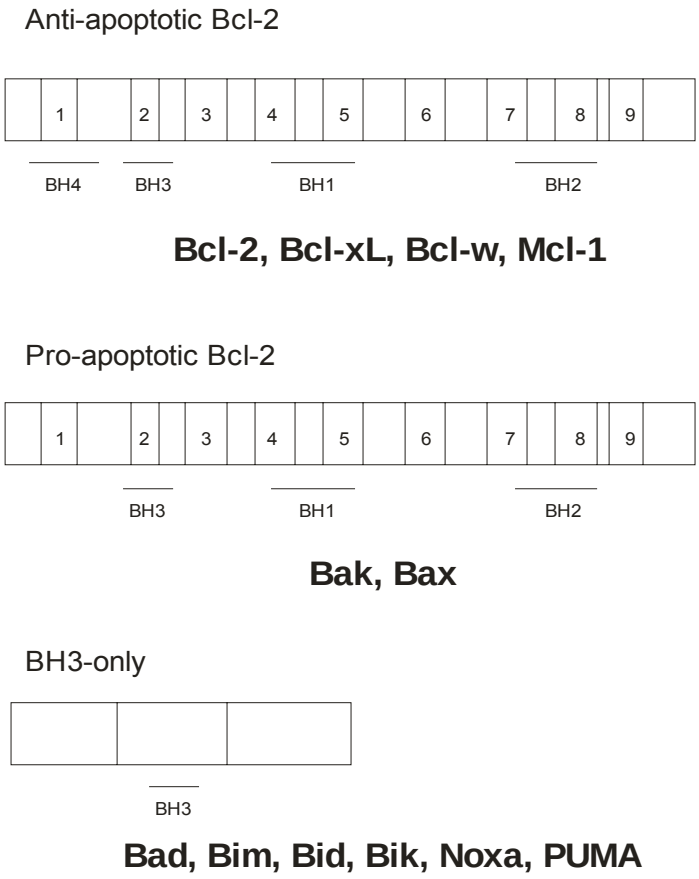


Figure 5: Bcl-2 homology domains. Based on the homology domains, the Bcl-2 family is divided into three functional groups. The anti-apoptotic group (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) contains four Bcl-2 homology domains (BH1,2,3,4). Pro-apoptotic group (Bax, Bak) contains three Bcl-2 homology domains (BH1,2,3). BH3-only group (Bad, Bim, Bid, Bik Noxa, PUMA) contains only one conserved domain (Bh3). Adapted image from *Trends in Cell Biology (2008) Vol 18. No. 4*

Bcl-x_L predominantly localizes to the mitochondrial membrane. Through its BH1-3 domains, Bcl-x_L binds to and sequesters pro-apoptotic molecules possessing the BH3 domain [131], such as Bax and Bak, and renders them inactive to initiate MOMP. Activation of Bax and Bak proteins leads to MOMP through conformational changes of the protein, inducing oligomerization and insertion into the outer mitochondrial membrane. This action forms pores through which proteins such as cytochrome *c* from the intermembrane space of the mitochondria can diffuse to the cytosol. Cytochrome *c* release can then trigger the assembly of apoptosomes and activate the caspase cascade [132-134]. Studies have shown that overexpression of Bcl-x_L can impede the oligomerization of Bax and Bak and prevents the release of cytochrome *c* by binding to those proteins [131, 135-137].

The expression of Bcl-x_L is regulated via three possible mechanisms: transcription [138, 139], alternative splicing [138] and translation [140]. Various studies have shown that mRNA stability also plays an important role in Bcl-x_L expression [141]. The stabilization is dependent on the 3'UTR region of the Bcl-x_L mRNA [139].

***3. Caspases*:** Caspases are **c**ysteine-rich **asp**artic-specific prote**ases**, that are some of the main effectors of apoptosis [142]. To date, there are 14 known mammalian caspases and all share a number of common features. For example, caspases are synthesized as inactive pro-enzymes, containing a prodomain followed by p20 and p10 subunits, and are activated by cleavage of the pro-enzymes [143]. All caspases can cleave at the Asp-Xxx bond, unique among mammalian proteases.

While overexpression of all 14 caspases results in cell death, not all of them are directly involved in cellular apoptosis [144]. The caspases can be divided into three groups based on their functions. First is the inflammatory caspase group that includes caspases 1, 4, 5, 11, 12, 13, and 14. These caspases induce inflammation, instead of cellular apoptosis. The second group includes the apoptotic initiator caspases. They have long prodomains containing either a death effector domain (DED) (caspase 8 and 10) or caspase activation and recruitment domain (CARD) (caspase 2 and 9), which are located upstream in the apoptotic pathway and are involved in the activation of other caspases. The last group contains the apoptotic effector caspases, which perform their function downstream in the apoptotic pathway by cleavage of proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) [145]. This executioner class (caspase 3, 6, and 7) is characterized by the presence of a short prodomain and is activated by upstream caspases [146].

Initiator and effector caspases are triggered by different mechanisms. The cellular death signal triggers the oligomerization of death adaptor proteins, such as Apaf-1 (intrinsic apoptotic adaptor protein) or FADD (extrinsic apoptotic adaptor protein). These death adaptor oligomers then induce the aggregation of pro-caspases. Previously, it was believed that the initiator caspases were autoproteolytically activated when brought close to each other (induced proximity model) [124]. This model was developed further into the proximity-induced dimerization model, in which the homodimerization of caspase 9 is promoted by the apoptososome due to the increased local concentration. In the same way, the DISC induces dimerization and subsequent auto-activation of caspase 8 [147, 148]. The death effector domains

(DED) of caspase 8 and 10 and the caspase activation and recruitment domains (CARD) of caspase 9 and 2 mediate their interactions with DED or CARD-containing adaptor proteins such as FADD or Apaf-1 [149]. Studies of knockout mice indicate that caspase 8 is required for all extrinsic (death receptor mediated) apoptosis, whereas caspase 9 is involved in the intrinsic (mitochondria-mediated) apoptotic pathway [150-152].

After the active initiator cleaves and activates effector pro-caspases, the activated effector caspases cleave various death substrates to induce cell death. Most caspases are triggered by proteolytic cleavage at two sites in the zymogens containing the Asp-X sites [153]. The caspase cascade is used by cells for the activation of the three downstream effector caspases, caspase 3, 6, and 7 [154]. In vitro studies also show that caspases 3 and 7 can be activated by caspase 6, 8, 9 and 10 [146]. These effector caspases are more abundant and active than the initiator caspases 8 and 9. Caspase 3 knockout mice display an apoptotic defect in both intrinsic and extrinsic pathway stimuli, suggesting that caspase 3 plays a crucial role as an executioner caspase [155, 156].

Pulmonary Epithelial and Endothelial Cell Apoptosis and Survival of Myofibroblasts in IPF

Apoptosis plays a key role in homeostasis to maintain a balance between cell proliferation and cell death. Repair following the lung injury involves the removal of proliferating mesenchymal and inflammatory cells from the alveolar airspace or

alveolar wall [157]. Failure to clear unwanted cells through apoptosis prolongs inflammation due to the release of unnecessary toxic elements from those cells, which subsequently results in pulmonary fibrosis. The key features of the pathobiology of IPF are the myofibroblast accumulation and activation [158]. Exaggerated apoptosis can also result in unbalanced tissue homeostasis and contribute to the pathogenesis. Increased apoptosis of alveolar epithelial cells (AEC) and endothelial cells may result in ineffective regeneration of a damaged lung, thus promoting a fibrotic tissue repair response.

There are two types of AECs that are morphologically and functionally different. The type I AECs are large elongated cells on the surface of the lung, which comprise the greater part of the epithelial component of the air-blood barrier (Figure 2) [159]. Type II AECs are cuboidal cells with rounded nuclei that have numerous cytoplasmic organelles, including lamellar bodies, which are the storage sites for pulmonary surfactant [159]. Both AEC types play an essential function in the respiratory task of the lung and are thought to be the main sites of lung damage in pulmonary fibrosis, causing interstitial fibrosis [160]. While the AEC in IPF appear to be injured with a high rate of apoptosis, the factors involved are not known and the mechanism of cellular apoptosis is still unknown. Recent studies demonstrated increased apoptosis in type II AECs in human patients with IPF [161]; increased expression of pro-apoptotic proteins and decreased expression of anti-apoptotic proteins were also reported [162].

While the AEC apoptosis in IPF is evident, the cause/effect relationship is still unclear. Animal model data suggest that excessive AEC apoptosis can lead to lung

fibrogenesis. Mice that were contacted with aerosolized activating anti-Fas antibody were associated with AEC apoptosis and the development of pulmonary fibrosis [15]. Transgenic overexpression of active transforming growth factor β-1 (TGFβ-1) on murine lung induced epithelial apoptosis, which resulted in mononuclear-rich inflammation, tissue fibrosis, and myofibroblast hyperplasia, and honeycombing, which is a classic pathological characteristic of fibrosis [163]. Furthermore, bleomycin-induced pulmonary fibrosis in murine model expressed AEC apoptosis, which was shown to be inhibited by caspase inhibitors [164].

While the causative factors and the mechanism of AEC apoptosis are still unknown, studies have shown that both the intrinsic [165] and extrinsic [166-168] apoptosis pathways are involved. Type II AECs express high levels of p53 and DNA fragments in IPF patients [7, 162]. Oxidative stress also has been proposed to induce AEC apoptosis. IPF patients showed evidence of oxidative damage by quantifying the formation of 8-hydroxy-deoxyguanosine in their lung tissues [169, 170]. It was previously thought that the reactive oxygen species (ROS) generation that induced AEC death in IPF was from the alveolar inflammatory cells [169, 171] but recent studies suggest that structural cells of the lung, especially the activated myofibroblasts, produce high levels of extracellular ROS which can induce cell injury and death of epithelial cells [172].

Epithelial-mesenchymal interactions are essential to the normal organogenesis. If there is dysregulated communication between these cellular components, it can result in the initiation of fibrosis [158]. Various factors can contribute to this dysregulated communication. A study was done with the advanced fibrotic human

lung, which demonstrated that AEC apoptosis occurring next to the α-smooth muscle actin-expressing myofibroblasts can cause dysregulated communication [8] and also cause the secretion of Ang II [75]. When the isolated myofibroblasts from IPF lung tissue were activated by TGF-β1, they released a significant amount of hydrogen peroxide which induced apoptosis of lung epithelial cells [172]. Furthermore, it has been shown that TGF-β1 itself can induce Fas-mediated apoptosis [173]. Thus, myofibroblast-secreted products such as Ang II, Fas and ROS can either directly or indirectly induce apoptosis of AEC.

Elimination of myofibroblasts by apoptosis is a crucial phase during normal cutaneous wound healing; abnormal processes during this phase can lead to fibrotic disorders [174]. There are very few studies on the apoptosis and survival signaling of fibroblasts/myofibroblasts of IPF and thus, there are is definite evidence that the resistance of myofibroblasts to apoptosis is responsible for the fibroblastic foci of IPF. Also, the reasoning behind how the mesenchymal cells can survive while the adjacent endothelial and epithelial cells submit to apoptosis in the same alveolar microenvironment is still unknown. However, in the human IPF studies, while the tissues showed an increased rate of apoptosis and high levels of apoptotic markers, adjacent fibroblasts/myofibroblasts showed virtually no apoptosis [8, 162]. Based on the study by Denton *et al.* [175], genetic alterations in mesenchymal cells might be sufficient to promote a fibrogenic tissue response. This study suggests that inherent differences of the cell types might be the determining factor of cellular apoptosis and survival.

Pulmonary endothelial cells are primarily involved in homeostasis and gas exchange in the lung. While several studies have provided evidence for endothelial cell apoptosis as an important event in pulmonary fibrosis, clear understanding of the signaling mechanism is still unknown [176]. Endothelial cells damage is connected with interstitial edema, leukocyte invasion and decreased gas exchange [177]. The functional or structural defect of endothelial cells may lead to breakdown or cell death of type II epithelial cells and may promote pulmonary fibrosis [40].

Bleomycin, a common drug used to generate animal models of lung injury and fibrogensis, was shown to induce significant endothelial apoptosis in mice [178]. When VEGF, an angiogenic factor that promotes the growth and survival of endothelial cells [179], inhibited TGF-β1-induced endothelial apoptosis, it also ameliorated fibrotic remodeling in a rat model of pulmonary fibrosis [180]. Yoshida *et al.* found that endothelial cells from human patients with IPF had decreased levels of cell survival signals, such as MAPK, and increased apoptotic cell death [181].

Pulmonary endothelial cells are also activated in a variety of inflammatory diseases, and their dysfunction is important in pulmonary fibrosis. Endothelial cells express inflammatory and fibrogenic cytokines, which is important in inflammatory infiltration, cellular growth and matrix synthesis [182].

Angiotensin II and Pulmonary Fibrosis

Angiotensin is a polypeptide vasopressor hormone generated by the catalytic action of renin, which is released mainly by the kidneys in reaction to low blood

volume or low sodium content in the body (Figure 6) [183]. Renal juxtaglomerular cells release renin when the juxtaglomerular apparatus decreases in the kidneys. Renin, also called angiotensinogenase, is a hydrolase that catalyzes cleavage of the leucine-leucine bond in angiotensinogen (a serum globulin) to form angiotensin I in the liver [183]. Angiotensin I acts mainly as a precursor to Ang II, which is a vasopressor and stimulator of aldosterone secretion by the adrenal cortex, and functions also as a neurotransmitter. Ang II is formed by the catalytic action of the hydrolase peptidyl dipeptidase A on the C terminal phenylalanine of angiotensin I [184-186]. When activating Ang II, peptidyl dipeptidase A is known as angiotensin converting enzyme (ACE), and when cleaving and inactivating kinins, it is known as kininase II.

The vasoactive polypeptide Ang II in human pathology has been primarily studied with regard to its role in blood pressure homeostasis and hypertension, though its function has recently been extended to a variety of chronic and progressive tissue scarring diseases and organ sclerosis, with an emphasis on the heart, lung, liver and kidney [184]. These systems are characterized by the local production of Ang II, which exerts paracrine and autocrine effects in the vicinity of its sites of activation. Tissue remodeling in all of these organs involves a coordinated interplay between activated fibroblast proliferation and the death of normal constituent cells, and it has

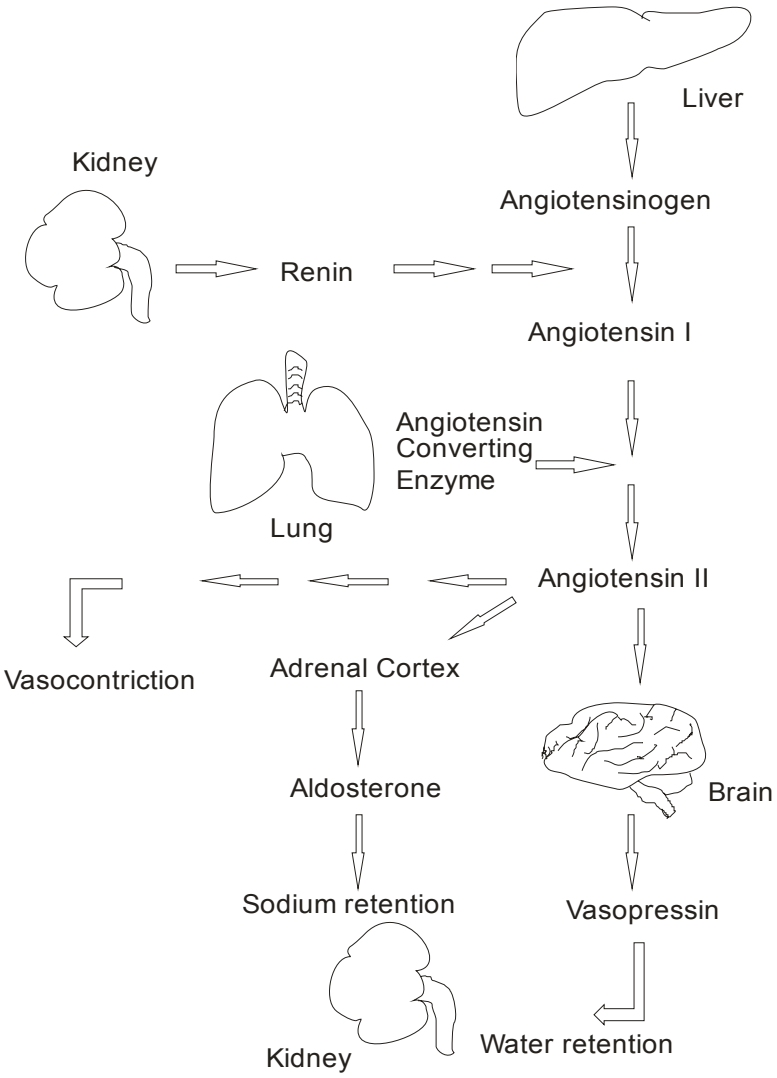


Figure 6: Angiotensin system. Angiotensinogen is produced and released from the liver. Angiotensin I is then formed by peptide cleavage of angiotensinogen by the renin enzyme that is produced in the kidney. Angiotensin I is convereted to angiotensin II by removing two terminal residues by the angiotensin converting enzyme, which is found mainly in the capillaries of the lung. Angiotensin II has various actions in the body, including vasoconstriction, aldosterone release, and vasopresin

been confirmed that Ang II is a potent inducer of apoptosis and the progression of fibrotic remodeling [187-190].

Increased findings in IPF patients, pulmonary fibrosis induced-animal models, and cell culture data have provided significant evidence for the importance of Ang II in the regulation of pulmonary epithelial and endothelial cell apoptosis in the progression of pulmonary fibrosis [191-193]. Biopsies of human fibrotic lung tissue show an increase in Ang II levels compared to normal healthy tissues [194, 195]. Studies by Konigshoff *et al.* [196, 197] demonstrated increased levels of Ang II and its receptors in patients with lung fibrosis. Patients with acute respiratory distress syndrome and pulmonary fibrosis, increased expression of ACE has also been shown [1, 198, 199]. Increased levels of Ang II were seen in fibrotic tissues in human studies, and *in vivo* studies using Ang II antagonists confirmed the role of Ang II in fibrosis in animal models [199]. Experimentally-induced lung fibrosis in rats and mice was inhibited by Ang II antagonists and ACE inhibitors [16, 200]. The blockade of Ang II- induced fibrosis was linked to inhibition of epithelial and endothelial cell apoptosis [16, 17, 200]. Immunohistochemistry experiments showed that epithelial cell apoptosis occurred in regions adjacent to activated myofibroblasts in the human lung [8]. Cultured fibroblasts taken from human lung tissue were shown to induce apoptosis in primary lung epithelial cells in an Ang II-dependent manner [75]. Fas and TNF-α have also been shown to be upregulated in fibrosis, resulting in epithelial cell apoptosis in a manner which requires *de novo* synthesis of Ang II [201, 202]. Ang II has been correlated with the increased expression of another important pro-fibrotic cytokine, transforming growth factor β (TGFβ), and remodeling events of pulmonary

fibrosis [203]. Recent studies have shown that Ang II is also strongly associated with tissue injury and fibrogenesis in circulatory organs [204] and lungs [205]. Together, these results indicate that Ang II plays a key role in lung epithelial and endothelial cell apoptosis during the development of lung fibrosis.

Additionally, ACE is found to be upregulated in response to tissue injury. For example, ACE mRNA and protein are upreguated during heart failure, vascular injuries as well as the bleomycin-induced pulmonary injuries [206, 207]. Physiologically, ACE may act as a tissue repair mediator early in the injury by activating Ang II, which can promote vascular inflammation, smooth muscle cell and fibroblast growth and deposition of ECM proteins [206, 208]. Increased ACE expression and Ang II production in damaged tissues have also been associated with remodeling and fibrosis [77, 209-212]. Investigations of the effects of ACE inhibitors on cardiac tissue reveal that ACE has a significant influence on cardiac remodeling [213]. Due to the fact that tissue remodeling entails a coordinated relationship between cell proliferation and death, it has been thought and confirmed that Ang II is a potent inducer of apoptosis [194, 195]. Capillary endothelial cells are considered the most effective producers of circulating Ang II by virtue of the ACE activity in the lumen of the pulmonary vascular lining [214, 215]. Animal studies using the ACE inhibitors captopril or lisinopril, attenuated fibrosis in the heart, kidney and lung by reducing fibroblast proliferation and preventing apoptosis of epithelial and endothelial cells [8, 17, 75, 210, 212, 216, 217]. ACE inhibitors have also been shown to slow myofibroblast growth in fibrotic tissue culture by reducing the Ang II production [77,

208, 218]. **While the connection between Ang II and fibrosis has been made, the signal transduction mechanism is still not understood.**

Although the exact extravascular concentration of Ang II in the lung is unknown, it is known that the levels increase in both plasma and lung tissue during injury [219, 220]. Lu *et al.* [221] showed that inflammatory cells, predominantly ED1+ macrophages, invading infarcted myocardium during the inflammatory phase of tissue repair, are implicated in the appearance of ROS at this site, which contributes to the inflammatory phase of repair post myocardial infarction. Upregulated expression of ACE and Ang II receptors by macrophages found at the infarct site insinuates a source for locally generated Ang II. More importantly, subsequent studies have found that endogenous or xenobiotic toxins can evoke an autocrine synthesis of Ang II [222]. It has been found that activation of Fas (APO1, CD95), a receptor known to be expressed and functional in the induction of apoptosis, stimulates AECs to synthesize Ang II, which in turn induces apoptosis [223]. Autocrine production of Ang II is also required for the apoptotic death of AECs in response to TNF-α [224]. **The finding that the autocrine generation of Ang II is required in the mediation of apoptotic cell death in both epithelial and endothelial cells may have major implications for the therapeutic management of acute lung injury and fibrogenesis.**

A study done in human umbilical vein endothelial cells [176] showed that purified Ang II could induce apoptosis with an EC_{50} of 100nM, a concentration well above the normal plasma Ang II concentration of 5-8pM [225] but a concentration which is believed to have physiological relevance in tissues where local Ang II production occurs [226]. The resulting apoptosis could be prevented by simultaneous

blockade of both angiotensin II receptor type 1 (AT1) and type 2 (AT2). Another study showed that in human coronary artery endothelial cells, Ang II could potentiate apoptosis induced by TNF-α [227]. **So far, the effect of Ang II-induced apoptosis has not been determined with primary cultures of pulmonary microvascular endothelial cells**.

Angiotensin II Signaling Pathways

The classes of Ang II receptors, AT1 and AT2, were first identified on a pharmacologic basis, followed by cloning of their cDNAs [224, 228, 229]. Both receptors are G protein coupled proteins with 7 transmembrane regions. The identification of the receptors led to the definition of new properties of Ang II, e.g., apoptosis, cell growth, and differentiation during development [196, 226]. Interestingly, Ang II exerts positive or negative survival effects depending on which subtype, AT1 or AT2, of receptors the peptide binds to as well as the cell types. AT2 receptors have been shown to be pro-apoptotic in fibroblasts, smooth muscle cells, HUVECs, and the rat pheochromocytoma cell line (PC12W), while AT1 was anti-apoptotic [176, 230, 231]. In contrast, Ang II-induced apoptosis in primary and transformed epithelial cells and coronary artery endothelial cells was shown to be mediated by the AT1 receptor [223, 227, 232]. The signaling pathways leading to apoptosis also proved to be cell type-dependent. In A549 and primary alveolar epithelial cells, AT1-mediated apoptosis was shown to be inhibited by the PKC inhibitor chelerythrine, but not by the broad tyrosine phosphatase inhibitor sodium

orthovanadate [232]. In contrast, in PC12W cells and HUVECs, AT2-mediated apoptosis was shown to require MAPK phosphatase-1 and -3, respectively, and subsequent Bcl-2 dephosphorylation [233, 234]. CHO-K1, COS-7, and neuroblastoma cells were shown to undergo apoptosis in response to the activation of the AT2 receptor [235]. **To date, the mechanism of Ang II on apoptosis has not yet been determined with primary cultures of pulmonary endothelial cells and will be addressed in the thesis.**

1. Angiotensin II Type I Receptor (AT1): Mitogenic and hypertrophic effects mediated by the AT1 subtype are illustrated by a wide variety of phosphorylation cascades. The extracellular N-terminal region of the AT1 receptors and the transmembrane helices contain binding sites for Ang II and play roles in Ang II-mediated activation of the receptor [236]. The extracellular region contains cysteine residues, which are implicated as binding and glycosylation sites [237].

The AT1 receptor is coupled via different G proteins to various signal transduction systems, including enzymes, such as phospholipase C-β1, phospholipase C-γ1, phospholipase A2, adenylate cyclase, tyrosine kinase, or ion channels (L-type calcium channel). The binding of Ang II to the AT1 receptors occurs at two affinity levels. The high affinity status corresponds to an active conformation of the receptor consisting of a ternary complex made of Ang II, receptor, and a G protein, whereas the low affinity status corresponds to a binary complex without a G protein. In the presence of guanylnucleotides, the G protein is dissociated from the receptor resulting in a decrease in ligand affinity. Site-directed mutagenesis experiments show that the

three intracellular loops and the C-terminal segment of the receptor contain the amino acid sequences which are related to G protein coupling [238].

The Ang II-AT1 complex is internalized followed by a reinsertion of the AT1 receptor into the membrane after release of the ligand. Internalization represents one of the mechanisms whereby target cells become desensitized in response to a repeated treatment with Ang II. This process depends on the phosphorylation of serine and threonine residues in the C-terminal intracellular region of the receptor [236]. Activation of the AT1 receptor leads to the phosphorylation of the platelet-derived growth factor receptor (PDGF-R) [239] and to stimulation of Ras-Raf-mitogen-activated protein (MAP) kinase [240, 241] , p70 S6 kinase [241] and Janus Kinase (JAK) [242, 243] signaling pathways, usually activated downstream of cytokine and growth factor receptors.

2. Angiotensin II Type II Receptor (AT2): The AT2 receptor, consisting of 363 amino acids, is a seven transmembrane domain protein; but in contrast to the AT1 receptor, its binding to G proteins is still debated and its physiological role is not completely understood [244]. The AT2 receptor is highly expressed in the fetal tissue, including skeletal system, brain, fetal aorta, adrenal medulla, heart, kidney, and lung but regresses after birth, suggesting the importance of AT2 in fetal development [245]. While the expression is limited in adults to few tissues, during pathological conditions, AT2 expression is increased in numbers of injured tissues, including pancreas, heart, kidney, adrenals, brain, and lung [245-250] and expressed during tissue remodeling

where the **AT2 receptor is thought to play a role in the apoptosis of smooth muscle cells and endothelial cells** [176, 251-255].

AT1 receptor mRNA and protein have been found to localize to vascular smooth muscle cells, macrophages and in the stroma underlying the airway epithelium relating to underlying fibroblasts. The AT1 receptor protein is not expressed in the epithelium although there is a low level of mRNA [203]. In contrast, AT2 receptor mRNA and protein have been observed in the epithelium, with strong staining on the bronchial epithelial cell brush border and also on many of the underlying mucous glands [203]. The AT2 receptor is also present on some endothelial cells [203]. There is a rising speculation that the AT2 receptor, which may antagonize the *in vivo* effects of AT1 on blood pressure and renal natriuresis [256], may also participate in the regulation of vascular cell growth [257-259].

In vitro, AT2 receptors mediate inhibition of cell proliferation in rat coronary endothelial cells [253] and in NIH3T3 fibroblasts stably expressing the rat AT2 receptor cDNA [222]. Through an AT2 receptor-mediated mechanism, rat pheochromocytoma PC12W cells [231] and neurite outgrowth in NG108-15 cells [260] underwent apoptosis. Moreover, via the AT2 receptor, Ang II has been implicated in the synthesis of the ECM protein collagen [261]. A study by Konigshoff *et al.* found that highly expressed AT2 mediates fibrosis signaling in the bleomycin-exposed lung of mice [199]. Using the AT2 antagonist PD123319, Waseda *et al.* decreased bleomycin-induced pulmonary fibrosis in mice [262].

AT2 receptor signaling is entirely distinct from that of the AT1 receptor. There are three possible transduction mechanisms for AT2 receptor signaling: (1) the

activation of protein phosphatases, (2) the activation of the NO/cGMP system, and (3) stimulation of phospholipase A2 with subsequent release of arachidonic acid [263].

There are three specific phosphatases identified which are stimulated upon AT2 activation: mitogen-activated protein kinase phosphatase 1 (MKP-1) [233, 264, 265], Src homology-2 domain-containing phosphatase 1/2 (SHP-1/2) [264-266], and protein phosphatase 2A (PP2A) [267]. MKP-1 dephosphorylates threonine- residues, while PP2A is an okadaic acid-sensitive serine/threonine phosphatase [263]. Signaling via activation of the SHP protein phosphatase is considered the main mechanism in AT2 receptor signaling, especially for the negative cross-talk of this receptor with growth factors and the AT1 receptor.

One major action of AT2 is to counteract growth factor-induced cell proliferation and to induce apoptosis [268]. Growth factors mediate their growth promoting actions primarily via tyrosine-kinase receptors and several additional kinase driven phosphorylation steps further down the respective signaling cascades. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) seem to play a key role in these phosphorylation signaling cascades. The AT2 receptor hinders these phosphorylation cascades by reversion of the phosphorylation steps through phosphatase activation. Dephosphorylation and subsequent inhibition of ERK1/2 has been shown downstream of AT2 activation and all three AT2 activated phosphatases (MKP-1, SHP, and PP2A) seem to be involved [233, 266, 267, 269-271]. Since these phosphatases reach their maximal activation at different time points and display divergent dephosphorylation specificities, they may interact with the ERK1/2 cascade at different time points [263]. Studies showed that the AT2-activated phosphatases

block ERK1/2-mediated activity [272, 273]. The inactivation of ERK1/2 seems to be a key event not only in AT2-mediated growth inhibition, but also in apoptosis, which could be dependent on the activation of MKP-1 and SHP and subsequently, on Bcl-2 dephosphorylation by MKP-1 and upregulation of Bax [230].

Activation of nitric oxide (NO) release with a subsequent increase in intracellular cGMP levels is one of very few signaling pathways which are shared by the AT1 and AT2 receptors [274]. An AT2-coupled increase in NO generation can be observed in vascular tissue of diverse origin such as cultured bovine aortic endothelial cells [275, 276], microvessels and coronary arteries from dogs [277], and isolated rat renal arteries [277, 278]. In some tissues, the AT2-induced increase in NO synthesis depends on the release of endogenous bradykinin. In the vasculature, this signaling cascade mediates vasorelaxation induced via AT2 receptors, a common result of NO production [279].

In vivo studies have shown that Ang II, via the AT2 receptor, also activates phospholipase A2 [280]. This results in the release of arachidonic acid and contributes to the activation of a $Na^+/HCO3^-$ symporter system, which regulates intracellular pH [281, 282]. In renal cells, arachidonic acid released upon AT2 stimulation and cytochrome P450 dependent metabolites generated from arachidonic acid have been shown to activate MAP-kinases and additional kinases further downstream of the Ras signaling pathway [283-285].

3. Other AT2 properties: ATBP50 is a Golgi membrane-associated protein which, on activation, binds to the cytoplasmic carboxy terminal of AT2 and regulates

the transport of the AT2 receptor to the cell membrane, thus modulating AT2 expression at the cell surface [286]. The level of receptor expression seems to be critical for the inhibition of proliferation and the induction of apoptosis, since the inhibitory effect mediated by AT2, at least in part, depends on the presence and density, but not on the extracellular activation of the receptor [287].

Ceramides are intracellular lipid second messengers involved in cytokine signaling. It has been shown that ceramides are *de novo* synthesized upon AT2 activation [288]. They are thought to mediate pro-apoptotic stimulus via activation of caspase 3 [288, 289]. Caspase 3 has been previously shown to be upregulated in response to AT2 activation and to subsequently initiate apoptosis [176].

AT2-Associated Proteins

1. G-protein subunits: Although the AT2 receptor is related to G-protein coupled receptors, the signaling mechanism of the receptor seems to involve unusual second messengers in addition to more classical G-protein coupled mediators [290]. In some cases, AT2 is coupled to $Gi_{\alpha 2}$- and $Gi_{\alpha 3}$- proteins [291, 292]. However, in a recent study by Feng *et al.* [235], activation of the tyrosine phosphatase SHP-1 seems to depend on the $G_{\beta\gamma}$-independent constitutive association of Gαs with SHP-1 and the AT2 receptor in the epithelial cell line of NIE 115 cells and transfected COS-7 cells (Figure 7). Using mutant receptors and $G_{\alpha s}$ protein inhibiting peptides, the study showed that SHP-1 activation is completely independent of G-protein activation. Also, the constitutive association of the isolated alpha subunit, and not the

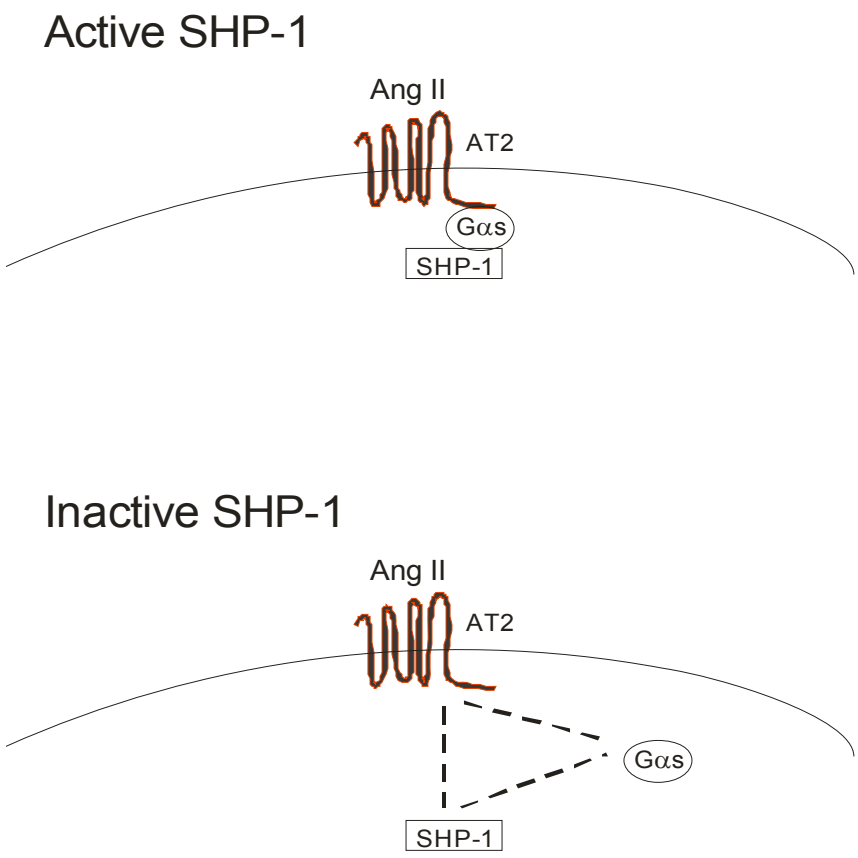


Figure 7: Activation of SHP-1. Feng *et al.* demonstrated in epithelial cells that SHP-1 activation is independent of G-protein activation. The constitutive association of the isolated alpha subunit, and not the hetero trimeric G-protein, is essential to the Ang II activation of SHP-1. In a non-activated state, SHP-1 is kept inactive through interaction of two SH2 domains with its catalytic domain. Upon physical interaction between the AT2 and Gαs, SHP-1 dissociates from the receptor and is activated by removal of the self-constraint imposed by the SH2 domain. In the absence of Gαs subunit, SHP-1 cannot be activated. The study concludes that the activation of SHP-1 requires the physical presence but an inactive form of Gαs. Feng *et al.* (2002) Proc Natl Acad Sci U S A. Vol. 99 No. 19

heterotrimeric G-protein, is essential to the Ang II activation of SHP-1. In an dormant state, SHP-1 is kept inactive through interaction of two SH2 domains with its catalytic domain. Upon physical interaction between the AT2 and Gαs, SHP-1 dissociates from the receptor and is activated by removal of the self-constraint imposed by the SH2 domain [235].

2. AMPK: AMP-activated kinase (AMPK) is a heterotrimeric kinase which functions as a metabolic regulator of cellular enzymes that are primarily involved in fat and carbohydrate metabolism, which regulate ATP conservation and synthesis [293]. When there is an increased ratio of AMP:ATP, AMPK is activated; increased AMPK activation is seen under conditions of stress, including hypoxia and ischemia. AMPK protein is formed by by α, β, and γ subunits; these three subunits play a specific role in the stability and the activity of AMPK. The γ subunit has four cystathionine beta synthase (CBS) domain which gives the AMPK the ability to detect the shift in the AMP:ATP ratio. The four CBS domain have binding site for AMP, also known as Bateman domain. As AMP binds to the Bateman domain, the γ subunit undergoes a conformational change which exposes the catalyic domain on the α subunit. In this catalytic domain, phosphorylation can take place at threonine-172 by AMPK kinase (AMPKK). This activation of AMPK then decreases the AMP:ATP ratio by turning off ATP-consuming pathways and turning on the ATP-generation pathways [293].

There have been few studies linking Ang II to the activation of AMPK [294, 295]. Using Ang II-infused rats, Hattori *et al.* [294] found that AMPK works as a

stimulator of the Ang II-induced cardiac fibroblasts. The study also showed that Ang II-activated AMPK leads to the increase of vascular smooth cell proliferation [294]. Feng *et al.* (unpublished finding) placed AMPK activation directly downstream of AT2 receptor activation. This finding coincides with Hattori's conclusion that AMPK phosphorylation and activation falls between Ang II receptor and downstream signals [296].

3. SHP-2: As mentioned above, SHP (1 and 2) phosphatase is one of the main proteins known to function downstream of AT2. It is characterized by two Src homology 2 (SH2) NH_2-terminal domains and a C-terminal protein-tyrosine phosphatase domain. Previous studies showed that SHP-1 is primarily expressed in epithelial and hematopoietic cells, but not in endothelial cells [297-301]. On the contrary, SHP-2 is expressed ubiquitously [302]. Various studies showed that the activation of SHP results from tyrosine phosphorylation [303-305]. In SHP-2, Tyr^{542} and Tyr^{580} are the proposed sites of phosphorylation [304, 305].

SHP proteins are involved in various cellular activities, such as trophic factor signaling, cell division, cell growth, cell differentiation, and cytoskeletal maintenance [306-312]. Specifically, studies have also linked the activation of SHP-2 to the apoptosis of epithelial, endothelial and hematopoietic cell and progression of fibrosis in the lung, heart, and the kidney [313-318].

Nucleolin

Nucleolin, also known as C23, is a nuclear phosphoprotein, abundantly expressed in exponentially growing cells and has been shown to be mainly localized in dense fibrillar regions of the nucleolus [319-322]. It has also been found on the cell surface [323-325] and in the cytoplasm [326, 327]. Nucleolin contains an import signal and can translocate between the cytoplasm and nucleus [328]. Schwab *et al.* showed that the cytoplasmic localization of nucleolin requires phosphorylation of the protein, while nuclear translocation is due to its dephosphorylation [329]. Studies demonstrate that nucleolin in the nucleus can induce apoptosis, while nucleolin in the cytoplasm has an anti-apoptotic effect [330, 331]. The downregulation of either form of nucleolin can inhibit cellular proliferation and induce apoptosis [332].

Nucleolin is a member of the ribonucleoprotein (RNP)-containing family of RNA-binding proteins. This mutilfunctional protein was shown to be involved in chromatin decondensation [333], ribosome maturation and assembly [334, 335], transcriptional regulation [336, 337], the transcription and processing of rRNA by RNA polymerase I [321, 338-342], cell proliferation [343], apoptosis [344], nuclear and cytoplasmic shuttling [345], mRNP assembly [346], and mRNA stability [347]. Phosphorylation of nucleolin by cdc2, CK2, and PKC-ζ have been connected to various cellular activities including cytokinesis, cell growth and proliferation, nucleogenesis, transcriptional repression and cytoplasmic-nucleolar transport of ribosomal components [348]. While the overall function of nucleolin has been studied in depth, the signal transduction pathways regulating nucleolin are not yet fully understood. A study by Westmark *et al.* presented that in peripheral blood

mononuclear cells, the upregulation of nucleolin mRNA required a phosphorylation of extracellular-regulated kinase (ERK) [349].

Nucleolin, composed of 707 amino acids, has three major domains [322]. Each nucleolin domain regulates different DNA, RNA, and protein processing. The amino-terminal domain, which contains α helical domain, is comprised of four acidic stretches. This domain controls the interaction with chromatin and modulates the nucleic acid binding activity of the protein [321, 322, 341, 350-353]. This region also contains phosphorylation sites for casein kinase II (CKII), cdc2, and other protein kinases; the phosphorylation of these sites stabilizes the nucleolin protein [354-356]. The carboxyl-terminal domain is defined as spaced Arg-Gly Gly (RGG) repeats interspersed with amino acids and functions to unfold the RNA secondary structure and also controls nucleolar localization [357-362]. In addition, this domain may also function to mediate protein-protein and protein-nucleic acid interaction [363].

The central globular domain is involved in pre-RNA recognition where the nucleolin can bind to two mutually exclusive RNA sequences [364]. This central domain contains four RNA binding domain (RBD) regions, where the protein can interact with the RNA [365, 366]. RBD1 and RBD2 with the 12 amino acid linker form the first binding site for the nucleolin recognition element (NRE), a hairpin that displays a conserved loop (U/G)CCCG(A/G) sequence. The NMR structure of nucleolin showed that RBD1 made contact with 6 RNA nucleotides and RBD2 contacted with 2 RNA nucleotides [367]. Furthermore, the protein linker showed sequence specific recognition of the RNA [368]. The second RNA-binding site is the evolutionarily conserved motif (ECM) that is a single stranded 11 nucleotide

sequence. In the nucleolin-EMC RNA complex, all four RBDs are required for binding [368].

1. Nucleolin-mediated mRNA stabilization of Bcl-x_L mRNA: In mammalian cells, the rate limiting step of mRNA degradation is controlled by polyadenylate [Poly(A)] RNase (PARN) that causes poly(A) deadenylation [369]. For mRNA degradation, the poly(A) tail must be shortened to ~30 to 60 nucleotides [370]. After deadenylation, hydrolysis of the 5' 7-methylguanosine cap takes place. This process allows 5'-3' exoribonuclease Xrn1 to degrade the decapped mRNA [371, 372]. However, studies have also shown that the primary degradation pathway can be mediated by exosomes, complexes made of at least ten 3'-5' exonucleases [369, 371, 372].

Many of the elements that regulate mRNA stability register to the 3'-untranslated regions (3'UTRs) of mRNA, including, IL-6, IL-2, cyclins, renin, c-myc, granulocyte, glucose transport 1, Cox-2, and ferritin [369, 373-375]. Prominently, a *cis*-element, AU-rich element (ARE) has been shown to be critical for the stability of mRNAs [376-379]. AREs typically contain multiple copies of the AUUUA pentamer and have a high content of U or A-U. AUUUA motifs are often associated with destabilization of mRNAs [370, 380]. Studies of various mRNAs suggest that the destabilizing effects of ARE and AUUA motifs can be increased or decreased by interactions with ARE-binding proteins [381, 382]. It is estimated that close to 10% of all mRNAs contain varying numbers of AREs [373, 375].

Each transcribed mRNA is associated with RNA-binding proteins to form specific ribonucleoprotein (RNP) complexes. Studies show that these complexes can determine the role in the mRNA, including stability [383, 384], nuclear and cytoplasmic localization [385-387] and alternative splicing [388]. There are two different mechanisms known by which the ARE can degrade and stabilize the mRNAs. The first possible mechanism is where the ARE-binding proteins physically interact with and recruit RNA decay machinery to accelerate RNA degradation. For example, K homology splicing regulatory protein (KSRP), Tristetraprolin and ARE/poly(U)-binding/degradation factor 1 (AUF1), well known destabilizing factors, accelerated mRNA degradation by recruiting the PARN and exosomes [371, 389]. On the other hand, proteins can bind to and interact with the RNA to stabilize and increase the mRNA half-life [390-392]. For example, HuR can stabilize and prevent mRNA degradation of numerous messages, such as those for p21, cyclins A and B1, β-casein, renin and Cox-2 through the ARE element [393].

The interaction of nuceolin with the RNA falls into the latter category of mechanisms, where the nucleolin protein binds to the 3'UTR mRNA to stabilize the message, and prevent mRNA from degradation (Figure 8). Nucleolin has been shown to bind to and stabilize various mRNAs including bcl-2, amyloid precursor protein, Gadd45, IL-2, β-globin and Bcl-x_L [141, 326, 391, 394, 395]. A study by Zhang *et al.* [141] ascertained that the nucleolin protein distinctively identifies the AU-rich elements (AUUUA) of the 3'UTR of the Bcl-x_L mRNA. Under UVA irradiation, the nucleolin protein dissociated from the Bcl-x_L mRNA and human keratinocyte cells

underwent apoptosis; however when the cells overexpressed the nucleolin protein, it stabilized

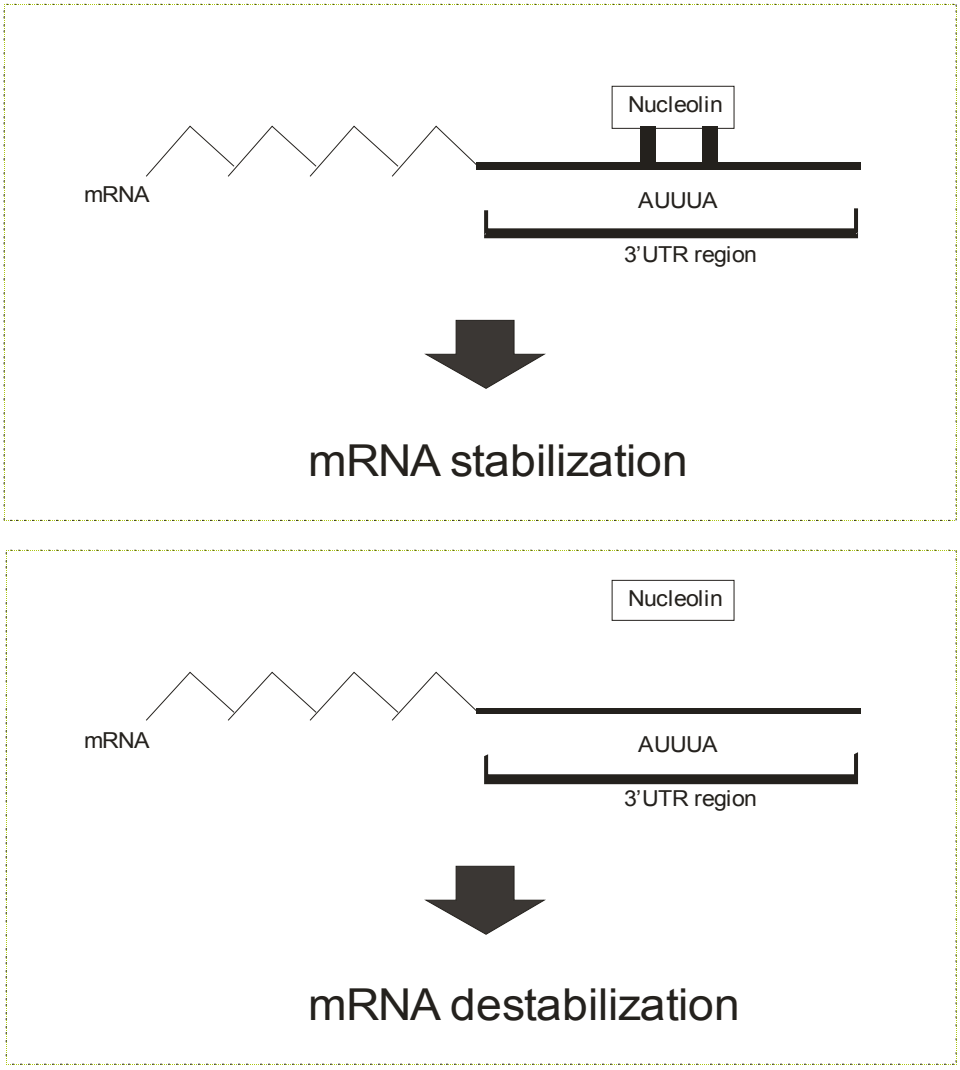


Figure 8: Nucleolin-mRNA interaction. Nucleolin protein has been shown to bind to the AU rich region of the 3'UTR region of the mRNA. The binding results in the stabilization of the mRNA, while the detachment causes destabilization and decrease in the mRNA decay.

the Bcl-x_L mRNA and cellular apoptosis was prevented. This study suggested that nucleolin protects the Bcl-x_L mRNA from nuclease degradation by RNA-nucleolin binding.

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a pleiotrophic peptide and growth factor that evokes a number of diverse responses in different cells and tissues. HGF, which was initially isolated from fibroblasts, stimulates the motility of epithelial cells and has been shown to have a wide range of effects on events which occur during the wound healing process [396-401]. HGF is structurally distinct from other growth factors and is mainly produced by mesenchymal cells [402, 403]. The mature HGF molecule is a heterodimer consisting of a 69 kDa α-chain and a 34 kDa β-chain (Figure 9) [399] . It contains a unique serine protease that catalyzes the enzymatic hydrolysis of the single-chain to elicit the mature heterodimeric form [398, 399, 404]. HGF contains an N-terminal hairpin loop and four kringle domains (K1, K2, K3, and K4) [405], each located on the α-subunit. The hairpin domain plays a role in mediating receptor-ligand binding [406], and the four kringle domains participate in the function and activation of the HGF receptor.

HGF is secreted by various cell types including pulmonary fibroblasts, first as an inactive 92 kDa single chain precursor form called pro-HGF [407, 408]. Once injury occurs in the tissue, the active and mature form of HGF is produced by proteolytic processing by HGF activator (HGFA) [407-409]. HGFA is first

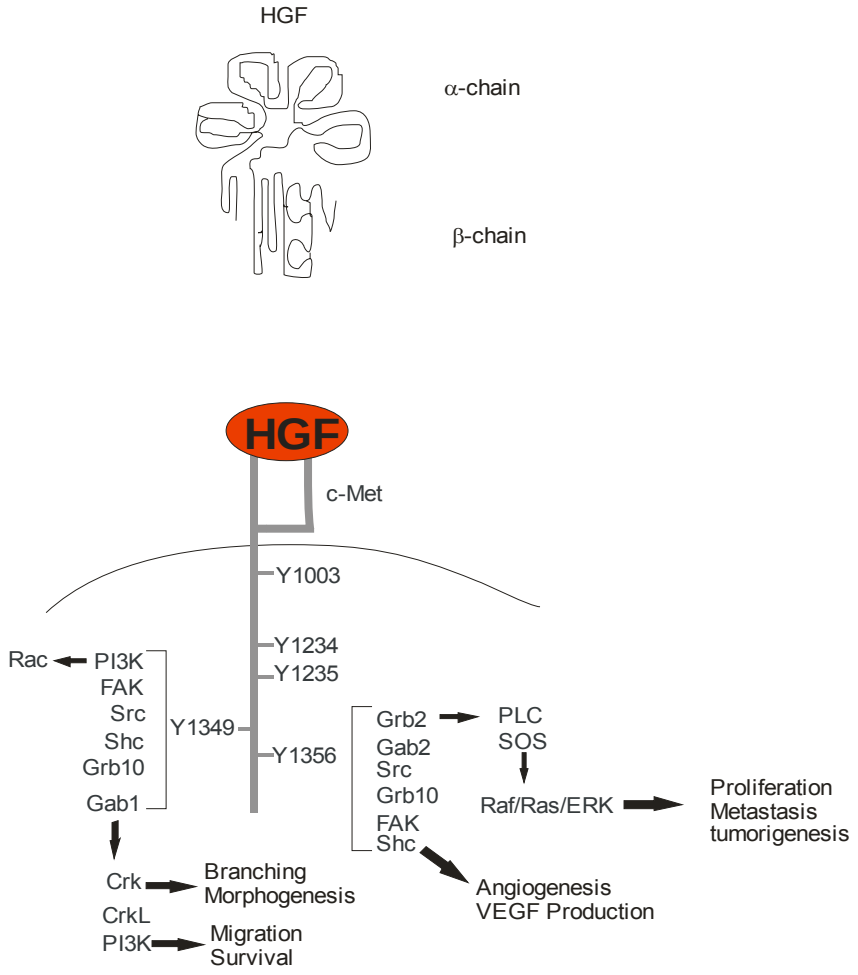


Figure 9: Hepatocyte Growth Factor and cMET receptor. Secreted by mesenchymal cells, HGF targets primarily on the epithelial and endothelial cells. It is secreted as a single inactive pol ypeptide and is cleaved by serine protease into a 69-kDa αchain and 34-kDa βchain. The αchain contains N-terminal domain (residues 31-127) followed by four kringle domains (K1-K4). The βchain resembles a serine protease in sequence but has no protease activity. A disulfide bond between the α and βchain produ ces the active, heterodimeric molecule. HGF are mediated by its receptor, cMET, a proto-oncogene product with an intracellular tyrosine kinase domain. c MET activation can mediate multiple signaling pathways, including proliferation, branching morphogeneiss, migration, and angiogenesis signals.

synthesized as an inactive precursor (96kDa) and is secreted by various cell types such as hepatocytes [407]. Pro-HGFA expression is shown to increase in the injured organ [408]. HGFA is then locally activated to the active form (34kDa), mainly by thrombin [410]. As mentioned above, the mature HGF is a heterodimeric molecule consisting of α-chain and β-chain held together by a disulfide bond. It is only in the heterodimeric form that HGF gains its activity, suggesting that the regulation of proteolytic activation of pro-HGF is crucial step in modulation of HGF biological activity [408, 411, 412]

HGF is required for a variety of biological functions including development and tissue repair in the adult. HGF stimulate cellular activities including growth, morphogenesis and motility. The diversity of function depends on specific target tissues and cell types. HGF mRNA has been shown to be elevated in both injured and noninjured tissues following damage to the kidney [413], gastrointestinal [414], lung [415], liver [416], myocardial tissue [417], and retinal epithelium [418]. Furthermore, HGF has been shown to ameliorate fibrosis as the result of injury in the lung [419-422], liver [423, 424], heart [216, 425, 426] and kidney [413, 427, 428].

HGF has been shown to play a key role in lung homeostasis, especially in the alveolar space [429, 430]. Experimental models of lung fibrosis using C57BL/6 mice, have shown that HGF acts as an anti-fibrotic factor [422]. Furthermore, HGF limited lung fibrosis *in vivo* after bleoymycin injury in rodents when given intravenously [422] or intratracheally [421]; on the other hand, an inhibitory anti-HGF antibody worsened pulmonary fiboris induced by bleomycin [296]. Simultaneous or delayed administration of HGF inhibits bleomycin-induced endothelial and epithelial cell

apoptosis and deposition of collagen [421, 422]. Similar findings have also been shown with hydrogen peroxide-induced rats, as an injection of HGF protein prevents lung injury [431].

HGF receptor cMet

The receptor for HGF is cMet, which is the product of the c-met protooncogene [432-434]. The receptor is a tyrosine kinase receptor consisting of two disulfide-linked subunits, a 50 kDa α-chain disulfide linked to a 145 kDa β-chain [406], with a single non-interrupted kinase domain which shares the homology with the src family of tyrosine kinases (Figure 9) [435-439]. The α-chain is exposed at the cell surface while the β-chain spans the cell membrane and possesses an intracellular tyrosine kinase domain [404, 434, 439, 440]. While HGF is a paracrine factor produced by cells of mesenchymal origin such as fibroblasts and macrophages, c-Met is expressed on epithelial cells [441] and nonepithelial cells including muscle cells [442], hepatic stellate cells [443], neurons [444, 445], microglial cells [446], and endothelial cells [447]. **Because the HGF receptor is located only on epithelial and endothelial cell types, but not on fibroblasts, it provides specificity for HGF-induced growth and anti-apoptotic activities.**

The binding of HGF to c-Met appears to be mediated primarily by the N-terminal, K1, and possibly K2 domains [434, 448-454]. HGF binds and induces tyrosine phosphorylation of the c-Met receptor, resulting in a ligand-dependent receptor homodimerization, which allows cross-phosphorylation of tyrosine residues

located on the intracellular portion of the cMet β-chain [439, 448-455]. The response elicited within the cell depends on which SH-2 containing protein binds to a specific phosphotyrosine on c-Met [456-458].

HGF-mediated signal transduction

cMet receptor expression is induced by a number of agents including HGF, phorbol myristate acetate, epidermal growth factor, conconavalin A, IL-1 and -6, TNF-α, tamoxifen, and steroidal hormones such as estrogen, progesterone, and dexamethasone [458-460]. Stimulation of c-Met by HGF results in the phosphorylation of intracellular signaling proteins containing Src homology domains such as phospholipase C-γ [457]. Phosphorylation of phospholipase C-γ (PLC γ) results in the hydrolysis of phosphatidylinositol bisphosphate to form diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) [406]. The formation of DAG and IP3 results in activation of distinct downstream signaling pathways of protein kinase C (PKC) and Ca^{2+} mobilization, respectively. PLC γ activation is required for growth factor-induced cell motility [461]. Cell proliferation results from phosphorylation of cMet followed by an activation of the mitogen activated protein kinase (MAPK) pathway in response to different intracellular signaling molecules [406]. Some of the adaptor proteins and direct kinase substrates found in this pathway are Grb2, Gab1, PI3K, phospholipase Cγ, Shc, Src, Shp2, Ship1 and STAT 3 [462, 463].

Gab and Grb2 are considered critical effectors and they interact with the receptor cMet directly. Following HGF binding to cMet and receptor

phosphorylation, Ras, the guanine-nucleotide binding protein, becomes stimulated by the guanine nucleotide exchange factor-catalyzed exchange of GDP for GTP [406]. Growth factors induce alteration in cell movement and cell shape by remodeling the actin cytoskeleton. Members of the Rho subfamily of small GTP-binding proteins mediate such alterations.

Another possible target of the HGF receptor kinase is the focal adhesion kinase (FAK) [464-466]. The FAK activation generates the formation of focal adhesions, an initial stage to increased cell motility and tissue invasion by transformed cells. The phosphorylation of paxillin, another focal adhesion protein, may also alter the adhesion of Met transformed cells [467]. HGF also inhibits anoikis, which is defined as the induction of apoptosis through suspension of cells, by triggering ERK and Akt kinase activations [468]. This activity may contribute to the anchorage independent growth of Met transformed cells. Signaling by integrins also plays a key role in HGF mediated tissue growth and cell invasion. The α6β4 integrin acts as a cofactor along with Met to participate in cell growth and proliferation [469]. Additionally to altering cell proliferation, motility, and cell adhesion, HGF adjusts cellular transcription through activation of STAT3, transcriptional factor known for anti-apoptotic and cellular growth properties [470, 471].

Chapter 4

Materials and Methods

Reagents- Angiotensin II was purchased from Bachem, Inc. (Torrance, CA). Cytochrome *c*, Bcl-2, and β-actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); normal rabbit IgG, anti-cleaved caspase 3, and SHP-2 antibodies were from Cell Signaling Technology (Danvers, MA). Dominant negative SHP-2, mutant C295S SHP-2, and wt-SHP-2 expression vectors, and $G_{\alpha s}$ inhibiting peptides and random peptides were gifts of Dr. Y. H. Feng, Uniformed Services University, Bethesda, MD [472, 473]. The Bax channel blocker and Bax inhibiting peptide V5 and control peptide were purchased from Tocris Bioscience (Ellisville, MO). The adenovirus Bcl-x_L expression vector, the adenovirus DN MEK, the GFP-nucleolin expression vector pEGF-C1, and Renilla-Bcl-x_L construct were gifts of Dr. Y.J. Suzuki (Georgetown University, Washington, DC), Dr. M. Kastan, St. Jude Children's Research Hospital, Memphis, TN [474], and Dr. G. Tim Bowden, University of Arizona, Tucson, AZ [141], respectively. Immortalized bovine pulmonary epithelial cells (PEpC) were a gift of Dr. David McClenahan, University of Northern Iowa, Cedar Falls IA [475].

Cell Culture- Bovine PAEC were from American Type Culture Collection (Manassas, VA). Passage 2-8 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA)/10% FBS (Gemini Bioproducts, Woodland, CA)/1% penicillin/streptomycin and 0.5% fungizone (Invitrogen). Immortalized bovine PEpC were cultured in Dulbecco's Modified Essential Medium (Invitrogen)/10% FBS (Gemini), 1% Glutamine, 0.005% epithelial growth factor, 0.01% insulin, 1% penicillin/streptomycin and 0.5% fungizone (Invitrogen). Cells were grown in 5% CO_2 at 37^{o}C in a humidified atmosphere culture incubator. PAEC

were grown to 80% confluence and placed in 0.1% FBS/RPMI overnight before treatment with agents.

Cell Lysate- To prepare whole cell lysates, confluent cells grown on 60 mm dishes were washed once with ice-cold PBS and collected using 100 µl lysis buffer 50 mM (Hepes, pH 7.4; 1% (v/v) Triton X-100; 4 mM EDTA; 1 mM sodium fluoride; 0.1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; and 10 µg/ml aprotinin). Then, cells were incubated for 15 minutes on ice, briefly vortexed, and insoluble materials were removed by centrifugation (14,000×g, 10 minutes at 4^{o}C). The supernatant was used for subsequent experiments.

Immunoprecipitation- Equal concentrations of protein from cell lysates were incubated with the primary antibody (1:1000 dilution). GammaBind Plus beads (1:100 dilution; Amersham Biosciences, Piscataway, NJ) were added and samples were incubated overnight on a rotator at 4^{o}C. The beads were then pelleted by centrifugation at 10,000×g for 10 minute at 4^{o}C, washed twice with lysis buffer containing protease and phosphatase inhibitors. To elute proteins, beads were resuspended in 25 µl Laemmli buffer incubated for 5 minute at 95^{o}C. The beads were removed by centrifugation at 14,000×g for 10 minutes. For immunoprecipitation of Bax protein, cells grown on 60 mm dishes were washed once with ice-cold PBS and collected using 100 µl of CHAPS buffer 50 mM Tris-HCl; 1mM EGTA; 1% (w/v) CHAPS; 10% glycerol; 50 mM sodium fluoride; 1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; and 10 µg/ml aprotinin). Then, cells were incubated for 15 minutes on ice, briefly vortexed, and insoluble

materials were removed by centrifugation (14,000×g, 10 minutes at 4°C). The supernatant was used for subsequent experiments.

Phosphastase Assay- SHP-2 immunoprecipitates on GammaBind beads were washed three times with cold lysis buffer (without Na_3VO_4) and three times with phosphatase buffer (50 mM Hepes, pH 7.4; 5 μg/ml aprotinin; and 1 μg/ml leupeptin). Phosphatase activity was assayed by 50 μl of reaction buffer (phosphatase buffer, pH 5.5; 1 mg/ml BSA; 5 mM EDTA; and 10 mM dithiothreitol). 50 μl of para-nitrophenyl phosphate (10 nM final concentration) was added at 37°C for 30 minutes. The reaction was stopped by the addition of 17 μl of 5 N NaOH, and the absorbance was measured at 405 nm.

Western blots- Whole cell lysates (10 μg of total protein) were subjected to SDS polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane (0.2 μm pore size). Membranes were blocked with 5% BSA in Tween 20 Tris Buffered Saline (TTBS, Tris Buffered Saline, 0.1% Tween 20) for 1 hour at ambient temperature. Membranes were then incubated overnight at 4°C with primary antibody (1:1000 dilution) in TTBS containing 0.5% BSA. Membranes were washed three times with TTBS for 10 minutes, then incubated with horse-radish peroxidase- labeled secondary antibody (1:1000 in TTBS) for 1 hour at ambient temperature. For protein detection, membranes were washed for 3 hours with TBS, incubated in ECL (Amersham), and analyzed on a FujiFilm Image Reader LAS-1000Pro (FujiFilm USA Inc., Valhalla, NY).

Mitochondria and mitochondria-free cytosolic protein extraction – Mitochondria extraction was performed using the Mitochondria Isolation Kit for Cultured Cells according to the manufacturer's protocol (Pierce, Rockford, IL).

Neutral Comet Assay- The neutral comet assay was used to measure double stranded DNA breaks as an indication of apoptosis [476]. After treatment, cells were embedded in 1% low-melting agarose and placed on comet slides (Trevigen, Gaithersburg, MD). Slides were placed in lysis solution (2.5 M NaCl, 1% Na-lauryl sarcosinate, 100 mM EDTA, 10 mM Tris base, 0.01% Triton X-100) for 30 minutes, then washed in 1×TBE buffer (0.089 M Tris; 0.089 M Boric acid; and 0.002 M EDTA, pH 8.0). Nuclei were electrophoresed for 10 minutes at 18 V in 1×TBE. Cells were then fixed with 75% ethanol for 10 minutes and air-dried overnight, stained with 1×Sybr® Green (Molecular Probes, Eugene, OR) or propidium iodide (Sigma-Aldrich, St. Louis, MO) and visualized with an Olympus FV500 confocal laser scanning microscope (Olympus Imaging America, Center Valley, PA) using 20× magnification at 478 nm excitation, 507 nm emission wavelengths for EGFP and Sybr Green and at 535 nm excitation, 617 nm emission wavelengths for propidium iodide. Cells were randomly selected per treatment group and assigned into type A, B or C comet categories, based on their tail moments. Type C comets were defined as apoptotic cells [477].

DNA laddering assay- Cells were harvested in the medium and pelleted at 1000 × g. Pellets were resuspended and incubated on ice in lysis buffer (1× Tris-EDTA, 0.2% Triton

at pH 8.0) for 15 minutes. Resuspended pellet was centrifuged (14,000 × g, 10 minutes at 4°C) and supernatant containing the fragmented DNA was collected. RNase A (final concentration at 60mg/ml) was added and incubated for 30 minutes at 37°C. SDS was added to a final concentration of 0.5% along with 150ug/ml of proteinase K and incubated 2 hours at 50°C. 0.1 volume of 5 M NaCl and 1 volume of ice cold isopropanol was added and samples were incubated on ice for 10 minutes. The samples were centrifuged at 13,000 × g for 15 minutes at 4°C. The DNA pellet was briefly dried and dissolved in 20ul of TE buffer, followed by electrophoresis (~2 hours, 20V) in 1.5% agarose.

Caspase assays. Caspase 8 and caspase 9 activities were measured using the Caspase-Glo® Assay according to the Cell-Based Assay protocol (Promega, Madison, WI). PAEC were grown in a 96-well white-walled cell culture plate (30,000-50,000 cells/well). Following treatments, 0.2 ml of Caspase-Glo® Assay Reagent, including the MG-132 Inhibitor, was added to each well containing 0.2 ml culture media, and incubated for 2 hours at room temperature on a rotating shaker. Luminescent signals were collected using a Dynex MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

RNA isolation and reverse transcription (RT)- Total RNA was obtained from bovine PAEC using the RNeasy kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-Free DNAse Set (Qiagen, Valencia, CA). RNA concentrations were determined spectroscopically at 260 nm (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE).

RNA (1.0 μg) was subjected to RT with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Semi-quantitative RT-PCR- 1μl of cDNA from the RT reaction was used for PCR reaction containing 0.4 μM each forward and reverse primer, 200 μM each dNTP, 1 U of i*Taq* DNA polymerase, and 1× PCR buffer (BioRad Laboratories Inc., Hercules, CA). PCR reactions were optimized for annealing temperatures using a temperature gradient in a BioRad iCycler. Reactions were carried out for 25 cycles using the following conditions: 95°C 1 minute; 60°C 45 seconds; 72°C 1.5 minutes. The last cycle extension was for 10 minutes at 65°C. PCR reactions were analyzed on a 1.5% agarose gel in Tris EDTA buffer and bands were visualized using ethidium bromide. All primer sequences are shown on the last page of materials and methods' section.

Quantitative real-time reverse transcription polymerase chain reaction (qPCR)- 0.2 μl of cDNA from the RT reaction was subjected to 20 μl qPCR with the primer pairs (Supplement Materials and Methods). qPCR was performed as recommended by the manufacturer (Applied Biosystems) in triplicates using 6 μM of each primer and 10 μl of SybrGreen PCR master mix (Applied Biosystems, Foster City, CA). PCR was run under the following conditions: activation of AmpliTaq Gold® Polymerase for 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Absence of non-specific amplification was confirmed by 2 % agarose gel electrophoresis. As an internal control mRNA level, α-tubulin was used. The comparative threshold cycle (Ct)

method was used to assess relative changes in mRNA levels. Data were collected from 4 to 6 experiments.

Cloning of EGFP-tagged histone H2B- Total mRNA was prepared from cultured primary human bronchial epithelial cells (Cell Applications, Inc.) and used for RT as described above. 2 µl cDNA was subjected to 50 µl PCR using the GC-Rich System (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions. PCR cycles were: 1× 94^{o}C, 3 minutes; 5× 94^{o}C 30 seconds, 55^{o}C 30 seconds, 72^{o}C 2 minutes; 22× 95^{o}C 30 seconds, 55^{o}C 30 seconds, 72^{o}C 1 minute; and 1× 72^{o}C 7 minutes. PCR product was analyzed by 1.5 % agarose gel electrophoresis, purified from the gel, and cloned into the *Bam* HI and *Kpn* I restriction sites of the pEGFP-N1 vector (BD Biosciences, San Jose, CA).

Determination of mRNA Half-life- Bovine PAEC were treated as indicated, then incubated with 5 µg/ml actinomycin D for time courses between 30 minutes and 8 hours. Total RNA was isolated and the level of Bcl-x_L mRNA was monitored by qPCR or by semi-quantitative RT-PCR (primers are listed above). Level of GAPDH mRNA was determined by gel electrophoresis and used for normalization of the semi-quantitative RT-PCR. ImageJ software was used for densitometry (http://www.uhnresearch.ca/facilities/wcif/index.htm).

Plasmid transfection - One day prior to transfection, cells were plated at 1.4×10^5 cells per well in a 12-well plate. One microgram of DNA per well was transfected using the

FuGENE 6 Transfection Reagent (Roche Applied Science), according to the manufacturer's instructions, in serum-free, antibiotic-free medium. The H2B-GFP reporter was co-transfected with the DN or mutant SHP-2 plasmids (or empty vector control), at a ratio of 1:2, total DNA concentration 1 μg. Cells were transfected for 6 hours, and then medium was replaced with 0.01% FBS medium with antibiotics. For luciferase assays, transfection mixtures contained a ratio of renilla-Bcl-x_L reporter construct to luciferase control vector (RSV-Luc) of 6:1 to normalize transfection efficiency.

Dual luciferase assay- Transfected cells were washed twice with cold PBS, lysed with passive lysis buffer, and assayed for firefly and Renilla luciferase activities using the Dual Luciferase Assay (Promega) according to the manufacturer's instructions in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA-IP: Cells were harvested by centrifugation at 1000 × g for 3 minutes and resuspended in 10 ml of PBS. RNA/protein complexes were cross-linked by adding of 1.0% formaldehyde (v/v) and incubating at room temperature for 10 minutes with gentle mixing. Cross-linking was quenched by adding glycine (pH 7.0, 0.125 mol/L final concentration), at room temperature for 5 minutes. Cells were washed twice with 10 ml PBS containing protease inhibitors and an RNase inhibitor and centrifuged 1000 × g for 3 minutes. Cell pellet was resuspended in 0.2 ml NP-40 buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40, protease inhibitors, RNase inhibitor) and incubated on ice for 10 minutes. Nuclei were pelleted by centrifugation at 1400 × g for 5 minutes at 4^{o}C. To fragment the mRNA, the supernatant was sonicated three times for 20 seconds each at output level 6 of a sonic

dismembrator, Model 100 (Fisher Scientific). Samples were cleared by centrifugation for 10 minutes, 13000 × g at 4°C, and the supernatant was collected and diluted 10-fold into IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, protease inhibitors, RNase inhibitor) to a final volume of 1 ml. A 1% aliquot was preserved as an input sample. Nucleolin antibody (1:1000 dilution) was added and samples were rotated overnight at 4°C. To collect immune complexes, 50 μL of Sepharose Beads were added and mixed for 2 hours followed by centrifugation at 60 × g for 2 minutes at 4°C. Immune complexes were washed for 5 minutes each with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and twice with TE buffer. Each wash was followed by 60 × g centrifugation for 1 minute at 4°C. Complexes were eluted in 500 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$, RNase inhibitor). NaCl was added to a final concentration of 200 mM then placed at 65°C for 2 hours to reverse crosslink. Next, 20 μl of 1 M Tris-Cl pH 6.5, 10 μl of 0.5 M EDTA, and 20 μg of Proteinase K was added to each sample and incubated at 42°C for 45 minutes. RNA was extracted using phenol:chloroform and 1 μl glycogen as a DNA-carrier. DNA was removed by DNase (Qiagen). RT-PCR was performed using 1 μl of the cDNA reaction for 25 cycles: denaturing was performed at 95°C for 1 minute, annealing for 45 seconds at 60°C, and polymerase reaction for 1.5 minutes at 72°C.

Ex-vivo Lung Explants- Mouse or rat lungs were used for the *ex-vivo* experiments. Mice and rats were euthanized with sodium pentobarbital or Fatal-plus. The surface of the

anterior chest wall and upper abdomen were sterilized with 70% ethanol. After the trachea was exposed, small nick was made to insert a 22 gauge needle with a short piece of polyethelene tube attached. Two lines were tied around the trachea to stabilize and to prevent the tube from slipping out. Through a midline abdominal incision the chest cavity was exposed and the animal was exsanguinated by dissecting the abdominal aorta. The right ventricle was punctured and the lungs were perfused with sterile PBS to remove the blood. Using aseptic technique, the trachea, lungs, and the heart was dissected from the animal.

To obtain lung slices for the ex-vivo culture, the lungs were inflated with 1% low melting point agarose dissolved in RPMI medium. The agarose was instilled as a liquid into the trachea using a syringe and fully inflated the lungs. The lungs were placed in a sterile cell culture plates and at 4°C for at least 30 minutes to solidify the agarose. The heart was then excised from the lung and each lobe of the lungs was embedded on a cutting board with 1% agarose to prevent any movement. The agarose-filled and embedded lungs were then chopped on a MclLwain tissue chopper (GeneQ Inc. Quebec, Canada) into 500 microns thick slices. The lung explants slices were incubated in a cell culture media containing 10% FBS, 1% penicillin/streptomycin and 0.5% fungizone for an hour in 37°C in a humidified chamber with 5% CO_2. Each lung slices were then transferred to 24 well cell culture plate with a specific treatment group for 16 to 24 hours in 37°C humidified chamber with 5% CO_2.

To obtain the morphology and immunohistochemistry of lung explants, the agarose inflated lungs were fixed by immersion in 10% buffered neutral formalin for 24 hours. The fixed lungs were paraffin embedded and stored till ready to use. Using SL-3000 Edge

Cryostat (HACKER Instruments & Industries Inc, Winnsboro, SC), lung slices of 10 to 20 micron sections were made. The slices were then processed using standard hisological techniques. Primary antibodies of cleaved caspase 3 (Cell Signaling Technology, Beverly MA) was used in final dilution of 1:200. Negative controls were done by omitting the respective primary antibodies.

Statistical Analysis- Means ± standard deviations (SD) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test. For three or more groups, statistical analysis was performed using one-way ANOVA, followed by the Bonferroni post-analysis, as appropriate; $p < 0.05$ was considered statistically significant. For mRNA half-life, linear regression was calculated and confidence intervals determined. Statistical software for all analysis was SigmaStat 3.1 (Point Richmond, CA)

PCR primers sequences:

α-tubulin:	Forward 5'-CTC CAT CCT CAC CAC CCA CAC
	Reverse 5'-CAG GGT CAC ATT TCA CCA TCT
Bcl-2 ARE	Forward 5'- TGC TTT TGA GGA GGG CTG CAC
	Reverse 5'- ACT GCC TGC CAC AGA CCA GC
Bcl-$x_{L:}$	Forward 5′-GGT ATT GGT GAG TCG GAT CG
	Reverse 5′-GCT GCA TTG TTC CCG TAG AG
Bcl-x_{L} ARE-1:	Forward 5'-CCA CGA CAA TTG AGG AAG GT
	Reverse 5'-ACC TTC CTC AAT TGT CGT GG
Bcl-x_{L} ARE-2:	Forward 5'-ACC TTC CTC AAT TGT CGT GG
	Reverse 5'-GGG GAA AAG GGT CAG AAA C
Bcl-x_{L} ARE-3:	Forward 5'-CGG GCT CTC TGC TGT ACA TAT T
	Reverse 5'-AGC ATC AGG CTG TTC GAT CT
GAPDH:	Forward 5'- GAA GCT CGT CAT CAA TGG AAA
	Reverse 5'- CCA CTT GAT GTT GGC AGG AT
Histone H2B:	Forward 5'-CGC GGA TCC TTA GCG CTG CTC TAC T TG GTG AC
	Reverse 5'-CCC GGA GGT ACC GCC ACC ATG CCA GAG CCA GCG AAG TCT CGT
SHP-1:	Forward 5'-CCC AGC CGC AAG AAC CAG GG
	Reverse 5'-CGC TGA GCA CGG ACA GCA CA
SHP2:	Forward 5'-CAC GGT GTG CCC AGT GAC CC
	Reverse 5'-GCT GCT GCA TCA GGA CCA CA

Chapter 5

Results

ANGIOTENSIN II-INDUCED APOPTOSIS REQUIRES SHP-2 REGULATION OF NUCLEOLIN AND BCL-X_L IN PRIMARY LUNG ENDOTHELIAL CELLS

Young H. Lee, Ognoon Mungunsukh, Rebecca L. Tutino, Ana P. Marquez, and Regina M. Day

Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD, 20814

Address correspondences to: Regina M. Day, PhD, Department of Pharmacology, C2023, 4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA. Fax: 301-295-3220. E-mail: rday@usuhs.mil

Running head: Angiotensin II Induces Intrinsic Apoptosis

Key words: intrinsic apoptosis, mRNA half-life, Bcl-x_L, nucleolin, SHP-2, Ang II

Abbreviations: FBS, Fetal bovine serum; Ang II, Angiotensin II; AT1, Angiotensin II type 1 receptor; AT2, Angiotensin II type 2 receptor; ARE, AU-rich element; BSA, Bovine serum albumin; $G_{\alpha s}$, α subunit of stimulatory heterotrimeric G protein; MOMP, Mitochondrial outer membrane permeabilization; PAEC, Pulmonary artery endothelial cells; PKC, Protein kinase C; SHP-2, SH2 domain-containing protein tyrosine phosphatase 2

Summary:

Angiotensin II (Ang II) is a key pro-apoptotic factor in fibrotic tissue diseases. However, the mechanism of Ang II-induced cell death in endothelial cells has not been previously elucidated. Using the neutral comet assay and specific receptor antagonists and agonists, we found that Ang II-mediated apoptosis in primary pulmonary endothelial cells required the AT2 receptor. Ang II caused cytochrome *c* release from the mitochondria concurrent with caspase 3 activation and DNA fragmentation, and apoptosis was suppressed by an inhibitor of Bax protein channel formation, implicating mitochondrial-mediated apoptosis. There was no evidence that the extrinsic apoptotic pathway was involved since caspase 9, but not caspase 8, was activated by Ang II treatment. Apoptosis required phosphoprotein phosphatase activation, and inhibition of the SHP-2 phosphatase blocked cell death. Reduced levels of anti-apoptotic Bcl-2 family members can initiate intrinsic apoptosis, and we found that Ang II treatment lowered cytosolic Bcl-x_L protein levels. Because the protein nucleolin has been demonstrated to bind Bcl-x_L mRNA and prevent its degradation, we investigated the role of nucleolin in Ang II-induced loss of Bcl-x_L. RNA immunoprecipitation experiments revealed that Ang II reduced nucleolin binding to Bcl-x_L mRNA in an AU-rich region implicated in the mRNA instability. Inhibition of SHP-2 prevented Ang II-induced degradation of Bcl-x_L mRNA. Taken together, our findings suggest that nucleolin is a primary target of Ang II signaling, and that Ang II-activated SHP-2 inhibits nucleolin binding to the Bcl-x_L mRNA, thus affecting the equilibrium between pro- and anti-apoptotic members of the Bcl-2 family.

Introduction:

The vasoactive peptide angiotensin II (Ang II) was originally studied for its role in blood pressure homeostasis, but abnormal expression of Ang II has been implicated in the development and progression of fibrotic organ diseases (Wynn, 2008). Blockade of Ang II signaling, either using inhibitors of angtiotensin converting enzyme to prevent Ang II maturation or using antagonists of Ang II cellular receptors, can abrogate the development of fibrosis in animal models in the lung, liver, kidney and the heart (Bataller et al., 2005; Dendorfer et al., 2005; Konigshoff et al., 2007; Sun, 2009; Wolf, 2008). In a number of fibrotic diseases, local synthesis of Ang II has been observed,and fibroblasts from the diseased tissues of human patients were found to generate Ang II (Bader, 2002; Sun et al., 2000; Wang et al., 1999). Two primary events are associated with the development and progression of fibrosis: 1) the transdifferentiation of fibroblasts into the activated fibroblast (myofibroblast) phenotype, that secretes abnormal extracellular matrix proteins such as collagens I and III (Wynn, 2008); and 2) the apoptosis of the normal epithelial and endothelial cells of the tissues (Marshall, 2003; Wang et al., 2000). Although much research has focused on epithelial cell apoptosis in fibrotic remodeling, several studies provided evidence for endothelial cell apoptosis in pulmonary fibrosis. Endothelial cell apoptosis has been identified in animal models of lung fibrosis (Zhang et al., 2007), and in samples from patients with idiopathic pulmonary fibrosis (Yoshida et al., 2002). Importantly, the use of VEGF to inhibit TGF-β1-induced endothelial apoptosis mitigated fibrotic remodeling in a rat model of pulmonary fibrosis (Farkas et al., 2009).

Ang II has been demonstrated to play a dual role in the development of fibrosis,

paradoxically inducing both the proliferation of scar tissue-producing myofibroblasts as well as the apoptosis of the primary epithelial and endothelial cells (Bataller et al., 2005; Uhal, 2002; Wolf, 2008). The contradictory actions of Ang II on the induction of growth versus apoptosis have been shown to be receptor subtype- and cell type-dependent. Ang II exerts its biological effects via the activation of two receptors known as angiotensin type 1 (AT1) and type 2 (AT2) receptors, both members of the seven transmembrane domain receptor family (Mogi et al., 2007). Ang II-induced apoptosis in primary and transformed epithelial cells and coronary artery endothelial cells was shown to be mediated by the AT1 receptor (Li et al., 1999; Papp et al., 2002). In contrast, AT2 receptor activation was shown to induce apoptosis in fibroblasts, smooth muscle cells, HUVECs, and PC12W cells, while AT1 activation resulted in proliferative and anti-apoptotic cellular responses in these cells (Cui et al., 2001; Dimmeler et al., 1997; Horiuchi et al., 1998; Yamada et al., 1996). Although many cells respond to AT2 receptor activation with decreased cell growth, AT2 receptor was shown to mediate proliferation in cardiomyocytes (D'Amore et al., 2005).

Ang II-induced signaling has also been demonstrated to be cell-type dependent, and the signaling specificity is believed to determine the biological response. Four major signal transduction pathways are activated by the AT receptors: (1) protein phosphatase activation; (2) the NO/cGMP activation; (3) phospholipase A2 activation, with subsequent release of arachidonic acid; and (4) protein kinase C (PKC) activation..Three specific phosphatases may be activated downstream of AT2: mitogen-activated protein kinase phosphatases (MKPs), that dephosphorylates threonine residues (Horiuchi et al., 1997); SH2 domain-containing phosphatases (SHP-1/2), a small family of tyrosine phosphatases (Nouet and Nahmias, 2000); and protein phosphatase 2A (PP2A), an okadaic acid-sensitive serine/threonine phosphatase

(Brechler et al., 1994). In A549 or primary alveolar epithelial cells, AT1-mediated apoptosis was inhibited by a protein kinase C (PKC) inhibitor, but not by tyrosine phosphatase inhibitors (Papp et al., 2002). In contrast, in PC12W cells and HUVEC cells, AT2-mediated apoptosis required MKPs, and subsequent Bcl-2 inactivation by dephosphorylation (Horiuchi et al., 1997; Rossig et al., 2002).

The mechanism of Ang II-induced apoptosis has not been previously determined in pulmonary endothelial cells. Here, we examined the mechanism of apoptosis by Ang II in primary cultures of bovine pulmonary artery endothelial cells. Ang II-induced apoptosis was found to be activated by the intrinsic (mitochondrial-dependent) apoptotic pathway. Moreover, we found that Ang II caused the destabilization and decay of Bcl-x_L mRNA by disassociation of the mRNA from the stabilizing protein nucleolin in a signaling pathway that required SHP-2.

Results:

Ang II-induced apoptosis requires the AT2 receptor. The local synthesis of Ang II has been demonstrated in lung fibrotic plaques, where it is produced by activated myofibroblasts and likely impacts the survival of other neighboring cells (Uhal, 2002; Wang et al., 1999). We investigated the effect of Ang II on bovine pulmonary artery endothelial cells (PAEC) using the neutral comet assay, which detects chromosomal breakdown as a function of apoptosis. Ang II (100 nM and 1 μM) induced 40-50% apoptosis within 24 hours, whereas 10 μM induced 60-70% apoptosis (Fig 1A). Higher concentrations of Ang II (100 μM) did not induce higher levels of apoptosis at 24 hours (Fig 1A). These findings were confirmed by monitoring DNA laddering induced by 100 nM and 10 μM Ang II at 24 hours (Fig 1B). A time course of

Ang II activity showed that significant apoptosis was detectable within 12 hours of treatment with 10 μM Ang II (Fig 1C).

Ang II receptors 1 and 2 (AT1 and AT2, respectively) are G-protein coupled receptors, and are the primary transducers of Ang II signaling. Pretreatment of PAEC with the AT2 antagonist PD123319 prior to exposure to 10 μM Ang II inhibited apoptosis as determined by neutral comet assay (Fig 2A). In contrast, no inhibition of Ang II-induced apoptosis was observed when cells were pretreated with telmisartan, an AT1 receptor antagonist (Fig 2B). The AT2 agonist CGP-42112A also induced apoptosis as determined by the neutral comet assay and DNA laddering assay (Fig 2C, D). The apoptotic effects of Ang II and CGP-42112A were reversed by the AT2 antagonist PD123319 (Fig 2E). Activation of caspase 3, a common effector caspase for both the intrinsic and extrinsic apoptotic pathways, was examined next. Results show that concentrations of Ang II as low as 0.1 μM induce the activation of caspase 3, which was blocked using the AT2 antagonist PD123319 (Fig 2F). These results indicate that Ang II–induced apoptosis is mediated by the AT2 receptor.

Ang II induces apoptosis via the intrinsic apoptotic pathway. The two canonical pathways of apoptosis in eukaryotic cells are the intrinsic (mitochondria-dependent) pathway and the extrinsic (death receptor mediated) pathway. Mitochondrial outer membrane permeabilization (MOMP) is a key event of the intrinsic apoptotic pathway. Western blots of mitochondria-free cell lysates showed the release of cytochrome *c,* as an indicator of MOMP, within 16 hours of Ang II or CGP42112A treatment (Fig 3A). Also at this time point, Ang II or CGP42112A activated caspase 3, the common effector caspase for both the intrinsic and extrinsic apoptotic pathways (Fig 3A). Mitochondrial-free cytosolic fractions contained

increased levels of activated caspase 3 and increased cytochrome *c*; however in the mitochondria, cytochrome *c* was only present in the untreated group, and both CGP 42112A and Ang II treatment cause cytochrome *c* release. The mobilization of the pro-apoptotic protein Bax to the mitochondria was monitored. With the Ang II treatment, the level of Bax protein increased in the mitochondria while the mitochondria-free cytosolic fraction showed significant decrease of the Bax protein levels (Fig S1). We therefore investigated the requirement of Bax activation for Ang II-induced apoptosis. Cell death was completely inhibited by either a Bax channel blocker (BCB) or a Bax inhibiting peptide (V-5), while a control peptide had no effect (Fig 3B).

We found that cell permeable inhibitor of caspase 3 (Z-DEVD-FMK) also completely blocked Ang II-induced apoptosis (Fig 3C). Caspase 3 is common to both the intrinsic and extrinsic pathways, so to further differentiate between the two apoptotic pathways, we examined the activation of caspase 9, the initiator caspase for the intrinsic pathway, and the activation of caspase 8, the initiator caspase for the extrinsic pathway. Caspase 9 was activated by both Ang II and the AT2 agonist CGP-42112A when compared to the control (Fig 3D), while caspase 8 was not activated by either treatment (Fig 3E).

Changes in MOMP can occur if the protein levels of anti-apoptotic members of the Bcl-2 family are reduced, especially Bcl-2 or Bcl-x_L, allowing mitochondrial pore formation by pro-apoptotic Bcl-2 family members, and a decrease in the ratio of Bcl-x_L:Bax (anti- to pro-apoptotic protein) is sufficient to induce apoptosis (Zhang et al., 2000). Treatment with Ang II and the AT2 agonist CGP-42112A significantly decreased Bcl-x_L protein levels (Fig 4A, upper panel). We did not detect any changes in Bcl-2 protein levels with Ang II treatment (Fig 4A, lower panel). To determine whether reduction of Bcl-x_L was critical for

Ang II-induced apoptosis, we ectopically expressed Bcl-x_L protein (Suzuki et al., 2007). Cells infected with an adenoviral vector encoding Bcl-x_L were protected from Ang II-induced apoptosis (Fig 4B) and prevented the activation of caspase 3 (Fig 4C). Infection with a GFP-expressing adenovirus had not effect on Ang II apoptosis or caspase 3 activation. Activated Bax permeabilizes the outer mitochondrial membrane, thereby committing cells to apoptosis. Bcl-x_L inhibits this process by binding directly to the activated Bax. Co-immunoprecipitation assays were performed to detect the amount of Bcl-x_L bound to Bax. The results demonstrated that Ang II treatment reduces the interaction of Bcl-x_L with Bax (Fig 4D). The input (whole cell lysates) was western blotted for Bcl-x_L with Bax as a control (Fig S2A). This control shows that the Bcl-x_L was reduced in the lysate in response to Ang II, but Bax levels were unchanged. The unbound fractions after immunoprecipitation of Bax were also western blotted for Bcl-x_L and Bax to show remaining unbound protein levels (Fig S2B). These data are consistent with Ang II-induced apoptosis through a mechanism which involves the down-regulation of Bcl-x_L.

SHP-2 activation is required for Ang II-induced apoptosis. The signaling pathways activated by Ang II through its receptors have been demonstrated to be cell type specific. Pharmacological inhibitors were used to identify signaling pathways originating from the AT2 receptor leading to apoptosis in PAEC. Ang II-induced apoptosis was blocked by the nonspecific inhibitor of tyrosine phosphatases, sodium orthovanadate (Fig 5A), while inhibitors of PKC (Chelerythrine chloride and Calphostin C) had no effect (Fig 5A,B). An inhibitor of the SH2 domain-containing protein tyrosine phosphatases (SHP) -1 and -2 (NSC-87877) blocked Ang II-induced apoptosis (Fig 5C).

Specific inhibitors are not available to differentiate between SHP-1 and SHP-2 isoforms (Yip et al., 2000). Previous studies showed that SHP-1 is primarily expressed in hematopoietic and epithelial cells (Chong and Maiese, 2007; Sankarshanan et al., 2007; Valencia et al., 1997; Yang et al., 1998; Yi et al., 1992). In contrast, SHP-2 is ubiquitously expressed (Bennett et al., 1996). To verify the SHP isoform expression profile in PAEC, RT-PCR was performed using primers specific for SHP-1 or SHP-2. Bovine pulmonary epithelial cells (PEpC) express both SHP-1 and SHP-2, but we found that bovine PAEC only express SHP-2 (Fig S3). The activation of SHP-2 in PAEC was confirmed by immunoprecipitation of SHP-2 followed by phosphatase assays. SHP-2 was activated within 1 minute of Ang II treatment (Fig 6A). Vogel *et al.* (Vogel et al., 1993) and Pazdrak *et al.* (Pazdrak et al., 1997) previously demonstrated that SHP-2 is tyrosine phosphorylated upon activation. Immunoprecipitation with anti-phosphotyrosine antibodies followed by western blotting for SHP-2 showed that SHP-2 was tyrosine phosphorylated in PAEC within 1 minute following Ang II treatment (Fig 6B).

To determine the requirement of SHP-2 for Ang II-induced apoptosis, we ectopically expressed either dominant negative (DN) SHP-2 or the SHP-2 mutant C459S (C459S-SHP-2), in which the phosphatase is inactive (Berchtold et al., 1998; Noguchi et al., 1994). PAEC were transiently transfected with wild type, DN or C459S-SHP-2 constructs, or empty vector control. Co-transfection of EGFP-tagged histone (H2BC-EGFP) was used for identification of transfected cells. Cells were treated with Ang II for 16 hours and apoptotic cells were identified using the neutral comet assay. Green fluorescent cells (cells positive for H2BC-EGFP expression) were scored for red comet tails stained with propidium iodide. Co-transfection of an empty vector confirmed that expression of histone H2BC-EGFP had no

effect on Ang II-induced apoptosis (Fig 6C). DN and C459S-SHP-2 expression significantly reduced apoptosis induced by Ang II (Fig 6C). Because SHP-2 is likely activated by association with AT2, over-expression of DN or mutant SHP-2 likely competes with endogenous wild type SHP-2 for binding with the receptor, thus preventing activation of the endogenous SHP-2. We also observed a significant increase in the level of basal apoptosis in cells over-expressing wild-type SHP-2 (Fig 6C), consistent with a pro-apoptotic role of SHP-2 in PAEC.

To determine the link between SHP-2 and Bcl-x_L protein reduction, PAEC were pretreated with a SHP phosphatase inhibitor (NSC-87877) prior to treatment with Ang II or the AT2 agonist CGP-42112A for 16 hours. Inhibition of SHP-2 preserved Bcl-x_L protein at basal levels (Fig 6D).

Ang II-induced apoptosis requires the $G_{\alpha s}$ protein as an adaptor. In transformed epithelial cells, SHP-1 is activated by the AT2 receptor in a mechanism that requires the physical association of $G_{\alpha s}$ protein (Feng et al., 2002). The activation of the G protein is not required for SHP-1 activation, suggesting that $G_{\alpha s}$ functioned as an adaptor molecule in this process. We investigated the requirement of $G_{\alpha s}$ for SHP-2 activation by AT2. A peptide corresponding to the last 11 amino acids of $G_{\alpha s}$ was shown by Feng *et al*. (2002) to inhibit the interaction between $G_{\alpha s}$ and AT2. The $G_{\alpha s}$ inhibiting peptide blocked activation of SHP-2 (Fig 7A) and reduced Ang II-induced apoptosis (Fig 7B). Feng *et al*. also showed that SHP-1 activation by cholera toxin A dissociated AT2-$G_{\alpha s}$ coupling by constitutively activating the $G_{\alpha s}$ protein. Activation of $G_{\alpha s}$ via cholera toxin A in PAEC also prevented both activation and tyrosine phosphorylation of SHP-2 protein by Ang II (Fig S4A,B). Cholera toxin A also

reduced Ang II-induced apoptosis by 70% (Fig. S4C, left panel). $G_{\alpha s}$-signaling activates the cAMP-dependent protein kinase (PKA). To determine whether activation of PKA affected Ang II-induced apoptosis, PAEC were treated with forskolin, to directly up-regulate cAMP and activate PKA, or with H89, to inhibit PKA. Forskolin treatment had no effect on Ang II-induced apoptosis (Fig S4C, middle panel), suggesting that the activation of adenylate cyclase, up-regulation of cAMP, and PKA activation were not the mechanism(s) by which cholera toxin A inhibited Ang II-induced apoptosis. H89 inhibition of PKA also failed to block Ang II-induced apoptosis (Fig S4C, right panel), suggesting that PKA is not required for apoptosis. Together, these findings suggest that $G_{\alpha s}$ acts as an adaptor for AT2-mediated activation of SHP-2 in endothelial cells, in a mechanism parallel to the activation of SHP-1 by AT2 in epithelial cells.

Ang II reduces the mRNA half-life of Bcl-x_L and reduces the binding of nucleolin to the Bcl-x_L mRNA 3'UTR. Our data indicated that Ang II reduced cytoplasmic Bcl-x_L protein levels within 16 hours of Ang II treatment. We investigated the effect of Ang II on Bcl-x_L mRNA, and found that mRNA levels were reduced within 4 hours of treatment (Fig 8A). Bcl-x_L mRNA levels are controlled by promoter regulation, by alternative splicing, and by the mRNA half-life (Boise et al., 1993; Kren et al., 1996; Li et al., 2004; Suzuki et al., 2007). Examination of the effect of Ang II on Bcl-x_L promoter activation showed no differences from basal levels, and alternatively spliced Bcl-x_L was not detected in our cells (data not shown). We therefore investigated effects of Ang II on the half-life of Bcl-x_L mRNA. Ang II caused a reduction in the half-life of Bcl-x_L mRNA, from ~7 hours in control cells to ~3 hours (Fig 8B).

Bcl-x_L mRNA stability is regulated by the 3'UTR, which contains AU-rich elements (AREs) that target the mRNA for degradation (Bachelor and Bowden, 2004). Nucleolin, a ubiquitously expressed 110 kDa multifunctional protein, has been shown to bind to the Bcl-x_L 3'UTR and stabilize the mRNA in the cytoplasm (Srivastava and Pollard, 1999; Zhang et al., 2008). We hypothesized that the Ang II-induced decrease in the Bcl-x_L mRNA half-life could be associated with decreased binding of nucleolin to the 3'UTR. RNA-IP experiments were performed to identify the nucleolin binding site and to determine the effect of Ang II signaling on nucleolin binding. We isolated Bcl-x_L mRNA fragments bound to nucleolin and used specific PCR primers to individually amplify the three AREs in the Bcl-x_L 3'UTR. The first ARE is located 164 nucleotides downstream of the stop codon, while the second and the third AU rich regions start at 317 and 1268 nucleotides downstream of the stop codon, respectively. We detected the highest binding affinity to the second ARE (Fig 9A). GAPDH, used as a negative control, is present in the input RNA sample but is not present after immunoprecipitation with nucleolin (Fig 9A). Ang II treatment of the PAEC significantly decreased nucleolin binding to the second ARE (Fig 9B). This data suggested that Ang II regulation of the Bcl-x_L mRNA half-live involves nucleolin binding to the second ARE of the 3'UTR.

Finally, we investigated the effect of Ang II on the stability of a reporter gene containing the Bcl-x_L mRNA 3'UTR. PAEC were transfected with a renilla-Bcl-x_L 3'UTR reporter construct (Bachelor and Bowden, 2004) and cells were either untreated or treated with Ang II. Renilla expression decreased significantly with the Ang II treatment (Fig 9C). We also found that inhibition of SHP-2 by NSC-87877 prior to Ang II treatment restored renilla expression to basal levels (Fig 9C). These results suggest that Ang II destabilized Bcl-

x_L mRNA by reducing nucleolin binding to the second ARE in the 3'UTR, via a signaling pathway that requires SHP-2.

Discussion:

The major finding of this study is that Ang II induces mitochondria-dependent apoptosis of primary pulmonary artery endothelial cells through the AT2 receptor in a signal transduction pathway leading to the down-regulation of Bcl-x_L protein through the destabilization of the Bcl-x_L mRNA. The balance between levels of pro- and anti- apoptotic proteins of the Bcl-2 family can determine mitochondrial membrane permeability (Zhang et al., 2000). Consistent with this we found that Ang II treatment reduced Bcl-x_L-Bax binding and caused translocation of Bax to the mitochondria. We also determined that the decreased half-life of the Bcl-x_L mRNA is associated with decreased nucleolin binding to the second ARE of the 3'UTR in a signaling pathway that requires SHP-2 phosphatase activation.

Ang II-induced apoptosis in PAEC required AT2, but not AT1 activation. Published findings provide conflicting evidence for the roles of AT1 and AT2 in cell proliferation and apoptosis. The contradictory effect of Ang II on cellular proliferation versus apoptosis has been hypothesized to be determined by cell type-specific expression of the receptors as well as differences in downstream signaling. The activation of SHP-1/2 protein phosphatases is considered one of the most critical downstream effects of Ang II, and is believed to mediate negative cross-talk between AT2 with AT1 and possibly with other growth factor receptors (Alvarez et al., 2008; Bedecs et al., 1997; Cui et al., 2001; Li et al., 2007; Marrero et al., 1998; Matsubara et al., 2001; Wu et al., 2004). In some cells, it has been demonstrated that Ang II treatment activates opposing signals through simultaneous AT1 and AT2 activation.

As an example of this, AT1 activation of ERK MAPK and Pyk kinase for cell survival is attenuated by AT2 activation of SHP-1 phosphatase in vascular smooth muscle cells (Cui et al., 2001; Matsubara et al., 2001). Our data indicate that SHP-2 is required for the Ang II-induced decrease of Bcl-x_L protein, and loss of anti-apoptotic Bcl-2 family members may also be the mechanism for Ang II-mediated inhibition of proliferative signaling from other receptors. Our data demonstrate that AT2 activation of SHP-2 also requires the presence of the inactive $G_{\alpha s}$ protein. In this complex containing AT2 and the $G_{\alpha s}$ protein, SHP-2 is rapidly phosphorylated with a time course (within 1 minute) that matches the enhancement of its phosphatase activity by Ang II. This suggests that the complex of the AT2 receptor, SHP-2, and $G_{\alpha s}$ may also contain a protein kinase. The identity of this kinase is currently under investigation in our laboratory.

We demonstrate that down-regulation of Bcl-x_L by Ang II is associated with decreased binding of nucleolin to the 3'UTR of Bcl-x_L mRNA. Nucleolin is a ubiquitously expressed, multifunctional RNA- and DNA-binding protein (Srivastava and Pollard, 1999). In the cytoplasm, nucleolin plays a role in cell survival through Bcl-2 and Bcl-x_L mRNA stabilization (Otake et al., 2007; Sengupta et al., 2004). Nucleolin binding to the 3' UTR of some mRNAs prevents AU-targeting of the mRNA for degradation (Zhang et al., 2008). However, upon dephosphorylation, nucleolin is translocated from the cytoplasm to the nucleus, where it is no longer available for mRNA stabilization (Schwab and Dreyer, 1997). Down-regulation of nucleolin, by siRNA or by agents that cause its degradation, is sufficient to induce growth arrest and apoptosis (Kito et al., 2003; Ugrinova et al., 2007). Our data indicate that SHP-2 phosphatase activity is necessary for Ang II-induced degradation of Bcl-x_L, and we hypothesize that the SHP-2 may directly affect the phosphorylation state of

nucleolin to either cause its translocation to the nucleus or to affect its stability. The phosphorylation site on nucleolin that is affected by SHP-2 has yet to be identified. Ang II-induced alterations in nucleolin protein levels, phosphorylation, and subcellular localization are currently under investigation in our laboratory.

Fibrotic remodeling involves both the transdifferentiation of normal fibroblasts to myofibroblasts and the induction of apoptosis in epithelial and endothelial cells (Friedman, 2004; Strieter, 2008; Tomasek et al., 2002; Wynn, 2008). Here we have identified a novel mechanism for Ang II-induced apoptosis in endothelial cells that involves SHP-2-induced destabilization of Bcl-x_L mRNA through reduction of nucleolin binding. The identification of specific pathways and mechanisms for apoptosis in fibrotic remodeling may provide novel targets for the mitigation of fibrotic diseases.

Materials and Methods:

Reagents- Angiotensin II was purchased from Bachem, Inc. (Torrance, CA). Cytochrome *c*, Bcl-2, and β-actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); normal rabbit IgG, anti-cleaved caspase 3, and SHP-2 antibodies were from Cell Signaling Technology (Danvers, MA). Dominant negative SHP-2, mutant C295S SHP-2, and wt-SHP-2 expression vectors, and $G_{\alpha s}$ inhibiting peptides and random peptides were gifts of Dr. Y. H. Feng, Uniformed Services University, Bethesda, MD (Berchtold et al., 1998; Noguchi et al., 1994). The Bax channel blocker and Bax inhibiting peptide V5 and control peptide were purchased from Tocris Bioscience (Ellisville, MO). The adenovirus Bcl-x_L expression vector, the GFP-nucleolin expression vector pEGF-C1, and Renilla-Bcl-x_L construct were gifts of Dr. Y.J. Suzuki (Georgetown University, Washington, DC), Dr. M. Kastan, St. Jude Children's Research Hospital, Memphis, TN (Takagi et al., 2005), and Dr. G. Tim Bowden, University of Arizona, Tucson, AZ (Zhang et al., 2008), respectively. Immortalized bovine pulmonary epithelial cells (PEpC) were a gift of Dr. David McClenahan, University of Northern Iowa, Cedar Falls IA (McClenahan et al., 2008).

Cell Culture- Bovine PAEC were from American Type Culture Collection (Manassas, VA). Passage 2-8 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA)/10% FBS (Gemini Bioproducts, Woodland, CA)/1% penicillin/streptomycin and 0.5% fungizone (Invitrogen). Immortalized bovine PEpC were cultured in Dulbecco's Modified Essential Medium (Invitrogen)/10% FBS (Gemini), 1% Glutamine, 0.005% epithelial growth factor, 0.01% insulin, 1% penicillin/streptomycin and 0.5% fungizone (Invitrogen). Cells were grown in 5% CO_2 at 37^{o}C in a humidified atmosphere culture incubator. PAEC were grown to 80% confluence and placed in

0.1% FBS/RPMI/1% penicillin/streptomycin and 0.5% fungizone overnight before treatment with agents.

Cell Lysate- Cells were washed with ice-cold PBS and lysed in 50 mM Hepes, pH 7.4; 1% (v/v) Triton X-100; 4 mM EDTA; 1 mM sodium fluoride; 0.1 mM sodium orthovanadate; 1 mM tetrasodium pyrophosphate; 2 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; and 10 µg/ml aprotinin. Lysates were incubated 15 minutes on ice, vortexed, insoluble materials were removed by centrifugation (14,000 × g, 10 minutes, 4^{o}C). For immunoprecipitation of Bax protein, cells were washed with cold PBS and lysed in CHAPS buffer (50 mM Tris-HCl; 1mM EGTA; 1% (w/v) CHAPS; 10% glycerol; 50 mM sodium fluoride; 1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; and 10 µg/ml aprotinin). Equal concentrations of protein from cell lysates were incubated with primary antibody (1:1000 dilution). GammaBind Plus beads (1:100 dilution; Amersham Biosciences, Piscataway, NJ) were added and samples were rotated at 4^{o}C overnight. The beads were centrifuged at 10,000 ×g for 10 minutes at 4^{o}C, washed twice with lysis buffer. To elute, beads were resuspended in 25 µl Laemmli buffer incubated for 5 minutes at 95^{o}C.

Phosphastase Assay- SHP-2 immunoprecipitates on GammaBind beads were washed three times with cold lysis buffer (without Na_3VO_4) and three times with phosphatase buffer (50 mM Hepes, pH 7.4; 5 µg/ml aprotinin; and 1 µg/ml leupeptin). Phosphatase activity was assayed by 50 µl of reaction buffer (phosphatase buffer, pH 5.5; 1 mg/ml BSA; 5 mM EDTA; and 10 mM dithiothreitol). 50 µl of para-nitrophenyl phosphate (10 nM final concentration) was added at 37^{o}C for 30 minutes. The reaction was stopped by the addition of 17 µl of 5 N NaOH, and the

absorbance was measured at 405 nm.

Western blots- Whole cell lysates (10 μg of total protein) were subjected to SDS polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane (0.2 μm pore size). Membranes were blocked with 5% BSA in Tween-20/Tris Buffered Saline (TTBS, Tris Buffered Saline, 0.1% Tween-20) for 1 hour at ambient temperature. Membranes were then incubated overnight at 4^{o}C with primary antibody (1:1000 dilution) in TTBS containing 0.5% BSA. Membranes were washed three times with TTBS for 10 minutes, then incubated with horse-radish peroxidase- labeled secondary antibody (1:1000 in TTBS) for 1 hour at ambient temperature. For protein detection, membranes were washed for 3 hours with TBS, incubated in ECL (Amersham), and analyzed on a FujiFilm Image Reader LAS-1000Pro (FujiFilm USA Inc., Valhalla, NY).

Mitochondria and mitochondria-free cytosolic protein extraction – Mitochondria extraction was performed using the Mitochondria Isolation Kit for Cultured Cells according to the manufacturer's protocol (Pierce, Rockford, IL).

Neutral Comet Assay- The neutral comet assay was used to measure double stranded DNA breaks as an indication of apoptosis (Kitta et al., 2001). After treatment, cells were embedded in 1% low-melting agarose (Sigma, St. Louis, MO) and placed on comet slides (Trevigen, Gaithersburg, MD). Slides were placed in lysis solution (2.5 M NaCl, 1% Na-lauryl sarcosinate, 100 mM EDTA, 10 mM Tris base, 0.01% Triton X-100) for 30 minutes, then washed in 1×TBE buffer (0.089 M Tris; 0.089 M Boric acid; and 0.003 M EDTA, pH

8.0). Nuclei were electrophoresed for 10 minutes at 18 V in 1×TBE. Cells were then fixed with 75% ethanol for 10 minutes and air-dried overnight, stained with 1×Sybr® Green (Molecular Probes, Eugene, OR) or propidium iodide (Sigma-Aldrich, St. Louis, MO) and visualized with an Olympus FV500 confocal laser scanning microscope (Olympus Imaging America, Center Valley, PA) using 20× magnification at 478 nm excitation, 507 nm emission wavelengths for EGFP and Sybr Green and at 535 nm excitation, 617 nm emission wavelengths for propidium iodide. Cells were randomly selected per treatment group and assigned into type A, B or C comet categories, based on their tail moments. Type C comets were defined as apoptotic cells (Krown et al., 1996).

DNA laddering assay- Cells were harvested in medium and pelleted at 1000 × g. Pellets were resuspended and incubated on ice in lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton at pH 8.0) for 15 minutes. Resuspended pellet was centrifuged (14,000 × g, 10 minutes at 4^{o}C) and supernatant containing the fragmented DNA was collected. RNase A (final concentration at 60 mg/ml) was added and incubated for 30 minutes at 37^{o}C. SDS was added to a final concentration of 0.5% along with 150 μg/ml of proteinase K and incubated 2 hours at 50^{o}C. 0.1 volume of 5 M NaCl and 1 volume of ice cold isopropanol was added and samples were incubated on ice for 10 minutes. The samples were centrifuged at 13,000 × g for 15 minutes at 4°C. The DNA pellet was briefly dried and dissolved in 20 μl of TE buffer, followed by electrophoresis (~2 hours, 20V) in 1.5% agarose.

Caspase assays. Caspase 8 and caspase 9 activities were measured using the Caspase-Glo® Assay according to the Cell-Based Assay protocol (Promega, Madison, WI). PAEC

were grown in a 96-well white-walled cell culture plate (30,000-50,000 cells/well). Following treatments, 0.2 ml of Caspase-Glo® Assay Reagent, including the MG-132 Inhibitor, was added to each well containing 0.2 ml culture media, and incubated for 2 hours at room temperature on a rotating shaker. Luminescent signals were collected using a Dynex MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

RNA isolation and reverse transcription (RT)- Total RNA was obtained from bovine PAEC using the RNeasy kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-Free DNAse Set (Qiagen, Valencia, CA). RNA concentrations were determined spectroscopically at 260 nm (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE). RNA (1.0 μg) was subjected to RT with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Semi-quantitative RT-PCR- 1μl of cDNA from the RT reaction was used for PCR reaction containing 0.4 μM each forward and reverse primer, 200 μM each dNTP, 1 U of i*Taq* DNA polymerase, and 1× PCR buffer (BioRad Laboratories Inc., Hercules, CA). PCR reactions were optimized for annealing temperatures using a temperature gradient in a BioRad iCycler. Reactions were carried out for 25 cycles using the following conditions: 95°C 1 minute; 60°C 45 seconds; 72°C 1.5 minutes. The last cycle extension was for 10 minutes at 65°C. PCR reactions were analyzed on a 1.5% agarose gel in Tris EDTA buffer and bands were visualized using ethidium bromide. All primer sequences are in the supplemental materials and method section.

Quantitative real-time reverse transcription polymerase chain reaction (qPCR)- 0.2 μl of cDNA from the RT reaction was subjected to 20 μl qPCR with the primer pairs (Supplement Materials and Methods). qPCR was performed as recommended by the manufacturer (Applied Biosystems) in triplicates using 6 μM of each primer and 10 μl of SybrGreen PCR master mix (Applied Biosystems, Foster City, CA). PCR was run under the following conditions: activation of AmpliTaq Gold® Polymerase for 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Absence of non-specific amplification was confirmed by 2% agarose gel electrophoresis. As an internal control mRNA level, α-tubulin was used. The comparative threshold cycle (Ct) method was used to assess relative changes in mRNA levels. Data were collected from 4 to 6 experiments.

Cloning of EGFP-tagged histone H2B- Total mRNA was prepared from cultured primary human bronchial epithelial cells (Cell Applications, Inc.) and used for RT as described above. 2 μl cDNA was subjected to 50 μl PCR using the GC-Rich System (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions. PCR cycles were: 1× 94°C, 3 minutes; 5× 94°C 30 seconds, 55°C 30 seconds, 72°C 2 minutes; 22× 95°C 30 seconds, 55°C 30 seconds, 72°C 1 minute; and 1× 72°C 7 minutes. PCR product was analyzed by 1.5 % agarose gel electrophoresis, purified from the gel, and cloned into the *Bam* HI and *Kpn* I restriction sites of the pEGFP-N1 vector (BD Biosciences, San Jose, CA).

Determination of mRNA Half-life- Bovine PAEC were treated as indicated, then incubated with 5 μg/ml actinomycin D for time courses between 30 minutes and 8 hours. Total RNA was isolated and the level of Bcl-x_L mRNA was monitored by qPCR or by semi-

quantitative RT-PCR (primers are listed above). Level of GAPDH mRNA was determined by gel electrophoresis and used for normalization of the semi-quantitative RT-PCR. ImageJ software was used for densitometry (http://www.uhnresearch.ca/facilities/wcif/index.htm).

Plasmid transfection - One day prior to transfection, cells were plated at 1.4×10^5 cells per well in a 12-well plate. One microgram of DNA per well was transfected using the FuGENE 6 Transfection Reagent (Roche Applied Science), according to the manufacturer's instructions, in serum-free, antibiotic-free medium. The H2B-GFP reporter was co-transfected with the DN or mutant SHP-2 plasmids (or empty vector control), at a ratio of 1:2, total DNA concentration 1 μg. Cells were transfected for 6 hours, and then medium was replaced with 0.01% FBS medium with antibiotics. For luciferase assays, transfection mixtures contained a ratio of renilla-Bcl-x_L reporter construct to luciferase control vector (RSV-Luc) of 6:1 to normalize transfection efficiency.

Dual luciferase assay- Transfected cells were washed twice with cold PBS, lysed with passive lysis buffer, and assayed for firefly and Renilla luciferase activities using the Dual Luciferase Assay (Promega) according to the manufacturer's instructions in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA-IP- PAEC were subjected to RNA-immunoprecipitation as previously described (Sun et al., 2006) with minor modifications (Supplemental Materials and Methods).

Statistical Analysis- Means ± standard deviations (SD) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test. For three or more groups,

statistical analysis was performed using one-way ANOVA, followed by the Bonferroni post-analysis, as appropriate; $p < 0.05$ was considered statistically significant. For mRNA half-life, linear regression was calculated and confidence intervals determined. Statistical software for all analysis was SigmaStat 3.1 (Point Richmond, CA).

Acknowledgements:

We thank Autumn J. Griffin for technical help and Bethanie L Morrison for constructive comments on the manuscript. This work was supported by NIH HL-073929 and a USUHS research grant to RMD, and by an American Heart Association predoctoral fellowship to YHL. Some of the authors are employees of the U.S. Government. This work was prepared as part of their official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government'. Title 17 U.S.C §101 defined a U.S. Government work as a work prepared by a military service member or employees of the U.S. Government as part of that person's official duties. The views in this article are those of the authors and do not necessarily reflect the views, official policy, or position of the Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, or the U.S. Federal Government.

Bibliography:

Alvarez, S. E., Seguin, L. R., Villarreal, R. S., Nahmias, C. and Ciuffo, G. M. (2008). Involvement of c-Src tyrosine kinase in SHP-1 phosphatase activation by Ang II AT2 receptors in rat fetal tissues. *J Cell Biochem* **105**, 703-11.

Bachelor, M. A. and Bowden, G. T. (2004). Ultraviolet A-induced modulation of Bcl-XL by p38 MAPK in human keratinocytes: post-transcriptional regulation through the 3'-untranslated region. *J Biol Chem* **279**, 42658-68.

Bader, M. (2002). Role of the local renin-angiotensin system in cardiac damage: a minireview focussing on transgenic animal models. *J Mol Cell Cardiol* **34**, 1455-62.

Bataller, R., Sancho-Bru, P., Gines, P. and Brenner, D. A. (2005). Liver fibrogenesis: a new role for the renin-angiotensin system. *Antioxid Redox Signal* **7**, 1346-55.

Bedecs, K., Elbaz, N., Sutren, M., Masson, M., Susini, C., Strosberg, A. D. and Nahmias, C. (1997). Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* **325 (Pt 2)**, 449-54.

Bennett, A. M., Hausdorff, S. F., O'Reilly, A. M., Freeman, R. M. and Neel, B. G. (1996). Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression. *Mol Cell Biol* **16**, 1189-202.

Berchtold, S., Volarevic, S., Moriggl, R., Mercep, M. and Groner, B. (1998). Dominant negative variants of the SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 (janus kinase 2) and induction of Stat5 (signal transducer and activator of transcription 5)-dependent transcription. *Mol Endocrinol* **12**, 556-67.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608.

Brechler, V., Reichlin, S., De Gasparo, M. and Bottari, S. P. (1994). Angiotensin II stimulates protein tyrosine phosphatase activity through a G-protein independent mechanism. *Receptors Channels* **2**, 89-98.

Chong, Z. Z. and Maiese, K. (2007). The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury. *Histol Histopathol* **22**, 1251-67.

Cui, T., Nakagami, H., Iwai, M., Takeda, Y., Shiuchi, T., Daviet, L., Nahmias, C. and Horiuchi, M. (2001). Pivotal role of tyrosine phosphatase SHP-1 in AT2 receptor-mediated apoptosis in rat fetal vascular smooth muscle cell. *Cardiovasc Res* **49**, 863-71.

D'Amore, A., Black, M. J. and Thomas, W. G. (2005). The angiotensin II type 2 receptor causes constitutive growth of cardiomyocytes and does not antagonize angiotensin II type 1 receptor-mediated hypertrophy. *Hypertension* **46**, 1347-54.

Dendorfer, A., Dominiak, P. and Schunkert, H. (2005). ACE inhibitors and angiotensin II receptor antagonists. *Handb Exp Pharmacol*, 407-42.

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J. and Zeiher, A. M. (1997). Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. *Circ Res* **81**, 970-6.

Farkas, L., Farkas, D., Ask, K., Moller, A., Gauldie, J., Margetts, P., Inman, M. and Kolb, M. (2009). VEGF ameliorates pulmonary hypertension through inhibition of endothelial apoptosis in experimental lung fibrosis in rats. *J Clin Invest* **119**, 1298-311.

Feng, Y. H., Sun, Y. and Douglas, J. G. (2002). Gbeta gamma -independent constitutive association of Galpha s with SHP-1 and angiotensin II receptor AT2 is essential in AT2-mediated ITIM-independent activation of SHP-1. *Proc Natl Acad Sci U S A* **99**, 12049-54.

Friedman, S. L. (2004). Mechanisms of disease: Mechanisms of hepatic fibrosis and therapeutic implications. *Nat Clin Pract Gastroenterol Hepatol* **1**, 98-105.

Horiuchi, M., Akishita, M. and Dzau, V. J. (1998). Molecular and cellular mechanism of angiotensin II-mediated apoptosis. *Endocr Res* **24**, 307-14.

Horiuchi, M., Hayashida, W., Kambe, T., Yamada, T. and Dzau, V. J. (1997). Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem* **272**, 19022-6.

Kito, S., Shimizu, K., Okamura, H., Yoshida, K., Morimoto, H., Fujita, M., Morimoto, Y., Ohba, T. and Haneji, T. (2003). Cleavage of nucleolin and argyrophilic nucleolar organizer region associated proteins in apoptosis-induced cells. *Biochem Biophys Res Commun* **300**, 950-6.

Kitta, K., Day, R. M., Ikeda, T. and Suzuki, Y. J. (2001). Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis. *Free Radic Biol Med* **31**, 902-10.

Konigshoff, M., Wilhelm, A., Jahn, A., Sedding, D., Amarie, O. V., Eul, B., Seeger, W., Fink, L., Gunther, A., Eickelberg, O. et al. (2007). The angiotensin II receptor 2 is expressed and mediates angiotensin II signaling in lung fibrosis. *Am J Respir Cell Mol Biol* **37**, 640-50.

Kren, B. T., Trembley, J. H., Krajewski, S., Behrens, T. W., Reed, J. C. and Steer, C. J. (1996). Modulation of apoptosis-associated genes bcl-2, bcl-x, and bax during rat liver regeneration. *Cell Growth Differ* **7**, 1633-42.

Krown, K. A., Page, M. T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K. L., Glembotski, C. G., Quintana, P. J. E. and Sabbadini, R. A. (1996). Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes: involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest* **98**, 2854-2865.

Li, C. Y., Chu, J. Y., Yu, J. K., Huang, X. Q., Liu, X. J., Shi, L., Che, Y. C. and Xie, J. Y. (2004). Regulation of alternative splicing of Bcl-x by IL-6, GM-CSF and TPA. *Cell Res* **14**, 473-9.

Li, D., Yang, B., Philips, M. I. and Mehta, J. L. (1999). Proapoptotic effects of ANG II in human coronary artery endothelial cells: role of AT1 receptor and PKC activation. *Am J Physiol* **276**, H786-92.

Li, J. M., Mogi, M., Tsukuda, K., Tomochika, H., Iwanami, J., Min, L. J., Nahmias, C., Iwai, M. and Horiuchi, M. (2007). Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1. *Mol Endocrinol* **21**, 499-511.

Marrero, M. B., Venema, V. J., Ju, H., Eaton, D. C. and Venema, R. C. (1998). Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. *Am J Physiol* **275**, C1216-23.

Marshall, R. P. (2003). The pulmonary renin-angiotensin system. *Curr Pharm Des* **9**, 715-22.

Matsubara, H., Shibasaki, Y., Okigaki, M., Mori, Y., Masaki, H., Kosaki, A., Tsutsumi, Y., Uchiyama, Y., Fujiyama, S., Nose, A. et al. (2001). Effect of angiotensin II type 2 receptor on tyrosine kinase Pyk2 and c-Jun NH2-terminal kinase via SHP-1 tyrosine phosphatase activity: evidence from vascular-targeted transgenic mice of AT2 receptor. *Biochem Biophys Res Commun* **282**, 1085-91.

McClenahan, D., Hellenbrand, K., Atapattu, D., Aulik, N., Carlton, D., Kapur, A. and Czuprynski, C. (2008). Effects of lipopolysaccharide and Mannheimia haemolytica leukotoxin on bovine lung microvascular endothelial cells and alveolar epithelial cells. *Clin Vaccine Immunol* **15**, 338-47.

Mogi, M., Iwai, M. and Horiuchi, M. (2007). Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors. *Arterioscler Thromb Vasc Biol* **27**, 2532-9.

Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y. and Kasuga, M. (1994). Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol Cell Biol* **14**, 6674-82.

Nouet, S. and Nahmias, C. (2000). Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab* **11**, 1-6.

Otake, Y., Soundararajan, S., Sengupta, T. K., Kio, E. A., Smith, J. C., Pineda-Roman, M., Stuart, R. K., Spicer, E. K. and Fernandes, D. J. (2007). Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA. *Blood* **109**, 3069-75.

Papp, M., Li, X., Zhuang, J., Wang, R. and Uhal, B. D. (2002). Angiotensin receptor subtype AT(1) mediates alveolar epithelial cell apoptosis in response to ANG II. *Am J Physiol Lung Cell Mol Physiol* **282**, L713-8.

Pazdrak, K., Adachi, T. and Alam, R. (1997). Src homology 2 protein tyrosine phosphatase (SHPTP2)/Src homology 2 phosphatase 2 (SHP2) tyrosine phosphatase is a positive regulator of the interleukin 5 receptor signal transduction pathways leading to the prolongation of eosinophil survival. *J Exp Med* **186**, 561-8.

Rossig, L., Hermann, C., Haendeler, J., Assmus, B., Zeiher, A. M. and Dimmeler, S. (2002). Angiotensin II-induced upregulation of MAP kinase phosphatase-3 mRNA levels mediates endothelial cell apoptosis. *Basic Res Cardiol* **97**, 1-8.

Sankarshanan, M., Ma, Z., Iype, T. and Lorenz, U. (2007). Identification of a novel lipid raft-targeting motif in Src homology 2-containing phosphatase 1. *J Immunol* **179**, 483-90.

Schwab, M. S. and Dreyer, C. (1997). Protein phosphorylation sites regulate the function of the bipartite NLS of nucleolin. *Eur J Cell Biol* **73**, 287-97.

Sengupta, T. K., Bandyopadhyay, S., Fernandes, D. J. and Spicer, E. K. (2004). Identification of nucleolin as an AU-rich element binding protein involved in bcl-2 mRNA stabilization. *J Biol Chem* **279**, 10855-63.

Srivastava, M. and Pollard, H. B. (1999). Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *Faseb J* **13**, 1911-22.

Strieter, R. M. (2008). What differentiates normal lung repair and fibrosis? Inflammation, resolution of repair, and fibrosis. *Proc Am Thorac Soc* **5**, 305-10.

Sun, B. K., Deaton, A. M. and Lee, J. T. (2006). A transient heterochromatic state in Xist preempts X inactivation choice without RNA stabilization. *Mol Cell* **21**, 617-28.

Sun, Y. (2009). Intracardiac renin-angiotensin system and myocardial repair/remodeling following infarction. *J Mol Cell Cardiol*.

Sun, Y., Zhang, J., Zhang, J. Q. and Ramires, F. J. (2000). Local angiotensin II and transforming growth factor-beta1 in renal fibrosis of rats. *Hypertension* **35**, 1078-84.

Suzuki, Y. J., Nagase, H., Wong, C. M., Kumar, S. V., Jain, V., Park, A. M. and Day, R. M. (2007). Regulation of Bcl-xL expression in lung vascular smooth muscle. *Am J Respir Cell Mol Biol* **36**, 678-87.

Takagi, M., Absalon, M. J., McLure, K. G. and Kastan, M. B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* **123**, 49-63.

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C. and Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* **3**, 349-63.

Ugrinova, I., Monier, K., Ivaldi, C., Thiry, M., Storck, S., Mongelard, F. and Bouvet, P. (2007). Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. *BMC Mol Biol* **8**, 66.

Uhal, B. D. (2002). Apoptosis in lung fibrosis and repair. *Chest* **122**, 293S-298S.

Valencia, A. M., Oliva, J. L., Bodega, G., Chiloeches, A., Lopez-Ruiz, P., Prieto, J. C., Susini, C. and Colas, B. (1997). Identification of a protein-tyrosine

phosphatase (SHP1) different from that associated with acid phosphatase in rat prostate. *FEBS Lett* **406**, 42-8.

Vogel, W., Lammers, R., Huang, J. and Ullrich, A. (1993). Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* **259**, 1611-4.

Wang, R., Ibarra-Sunga, O., Verlinski, L., Pick, R. and Uhal, B. D. (2000). Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol* **279**, L143-51.

Wang, R., Ramos, C., Joshi, I., Zagariya, A., Pardo, A., Selman, M. and Uhal, B. D. (1999). Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. *Am J Physiol* **277**, L1158-64.

Wolf, G. (2008). Novel aspects of the renin-angiotensin-aldosterone-system. *Front Biosci* **13**, 4993-5005.

Wu, L., Iwai, M., Li, Z., Shiuchi, T., Min, L. J., Cui, T. X., Li, J. M., Okumura, M., Nahmias, C. and Horiuchi, M. (2004). Regulation of inhibitory protein-kappaB and monocyte chemoattractant protein-1 by angiotensin II type 2 receptor-activated Src homology protein tyrosine phosphatase-1 in fetal vascular smooth muscle cells. *Mol Endocrinol* **18**, 666-78.

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol* **214**, 199-210.

Yamada, T., Horiuchi, M. and Dzau, V. J. (1996). Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci U S A* **93**, 156-60.

Yang, J., Liang, X., Niu, T., Meng, W., Zhao, Z. and Zhou, G. W. (1998). Crystal structure of the catalytic domain of protein-tyrosine phosphatase SHP-1. *J Biol Chem* **273**, 28199-207.

Yi, T. L., Cleveland, J. L. and Ihle, J. N. (1992). Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol Cell Biol* **12**, 836-46.

Yip, S. S., Crew, A. J., Gee, J. M., Hui, R., Blamey, R. W., Robertson, J. F., Nicholson, R. I., Sutherland, R. L. and Daly, R. J. (2000). Up-regulation of the protein tyrosine phosphatase SHP-1 in human breast cancer and correlation with GRB2 expression. *Int J Cancer* **88**, 363-8.

Yoshida, K., Kuwano, K., Hagimoto, N., Watanabe, K., Matsuba, T., Fujita, M., Inoshima, I. and Hara, N. (2002). MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. *J Pathol* **198**, 388-96.

Zhang, H., Lawson, W. E., Polosukhin, V. V., Pozzi, A., Blackwell, T. S., Litingtung, Y. and Chiang, C. (2007). Inhibitor of differentiation 1 promotes endothelial survival in a bleomycin model of lung injury in mice. *Am J Pathol* **171**, 1113-26.

Zhang, J., Tsaprailis, G. and Bowden, G. T. (2008). Nucleolin stabilizes Bcl-X L messenger RNA in response to UVA irradiation. *Cancer Res* **68**, 1046-54.

Zhang, L., Yu, J., Park, B. H., Kinzler, K. W. and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. *Science* **290**, 989-92.

Figure Legends

Figure 1. Ang II induces apoptosis in PAEC. A: PAEC were treated with Ang II for the indicated concentration for 24 hours before apoptosis detection using the neutral comet assay. B: PAEC were treated with Ang II (0.1 or 10 μM) for 24 hours. DNA was used in DNA laddering assays. Base pair standards for DNA fragment sizes are indicated. C: PAEC were treated with Ang II (10 μM) for the indicated times before assaying apoptosis using the neutral comet assay. Bar graphs show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 4. All experiments were repeated at least 4 times.

Figure 2. Ang II-induced apoptosis in PAEC requires the type 2 receptor. A,B: PAEC were treated with (A) the AT2 antagonist PD123319 (50 μM), or (B) the AT1 antagonist telmisartan (1 μM) for 20 minutes prior to the addition of Ang II (10 μM) for 24 hours. Apoptosis was determined using the neutral comet assay. C: PAEC were treated with either Ang II (0.1 or 10 μM), or AT2 agonist CGP-42112A (CGP, 0.01 or 10 μM) for 24 hours before determining apoptosis using the neutral comet assay. D: PAEC were either untreated (control, C) or treated with CGP-42112A (CGP, 0.01 or 10 μM) for 16 hours before purification of DNA for DNA laddering analysis. Representative results are shown from 3 experiments. E: PAEC were treated ± the AT2 antagonist PD123319 (50 μM) for 20 minutes prior to treatment with Ang II (0.1 or 10 μM) or the AT2 agonist CGP-42112A (CGP, 0.01 or 10 μM). F: PAEC were treated with Ang II (0.1 μM) ± pretreatment with the AT2 antagonist PD123319 (50 μM) for 20 minutes. After

16 hours, cell lysates were prepared and western blotted for the activated form of caspase 3. Blots were stripped and probed for β-actin as a loading control. Graph to the bottom shows the average results of caspase 3 band densitometry, normalized to β-actin, from 3 experiments. For all bar graphs, data show means, ± S.D. * indicates statistical significance from control, p=0.05, n = 3. † indicates statistical significance from Ang II treatment alone. Experiments were repeated at least 3 times.

Figure 3. Ang II induces the intrinsic pathway of apoptosis in PAEC. A: PAEC were treated with the indicated concentrations of Ang II or CGP-42112A (CGP) for 16 hours before the preparation of mitochondria-free cell fractions (cytosol) or mitochondria. Lysates were western blotted for cytochrome *c* or activated caspase 3. Blots were stripped and reprobed for β-actin as a loading control for cytosol or for COX4, a marker for mitochondria. Densitometry results normalized to β-actin or COX4 are shown in lower panels. B: PAEC were treated with the Bax channel blocker (BCB, 10 μM, left panel), or the Bax inhibitory peptide V5 (100 μM, right panel) or a Bax control peptide (100 μM) for 20 minutes prior to the addition of Ang II (10 μM) for 16 hours. Neutral comet assays were then performed and the percent apoptosis was determined. C: PAEC were pretreated with the caspase 3 inhibitor Z-DEVD-FMK (10 μM) before treatment with 10 μM Ang II for 16 hours. Neutral comet assays were performed and the percent apoptosis was determined. D,E: PAEC were treated with 10 μM Ang II or AT2 receptor agonist CGP-42112A (CGP, 10 μM) for 16 hours and assayed for caspase 9 (D) and 8 (E). TNFα (1 μg/ml) treatment for 16 hours was used as a positive control for caspase 8 activation; cytochrome *c* (10 μg/ml) was used as a positive control for activation of

caspase 9. For all bar graphs, data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Figure 4. Ang II induces apoptosis in PAEC by suppressing the Bcl-x_L protein levels. A: PAEC were treated with Ang II (10 μM) or AT2 agonist CGP-42112A (CGP, 10 μM) for 16 hours. Mitochondria-free cytosolic extracts were used for western blot of Bcl-x_L. Blots were stripped and reprobed for β-actin. Densitometry results normalized to β-actin are shown to the right. B,C: PAEC were infected with adenovirus (Adv) GFP or Bcl-x_L for 48 hours, followed by 10 μM Ang II treatment for 24 hours. B) Neutral comet assays were performed to detect apoptotic cells. Percentage of apoptotic cells were then determined. C) Lysates were used for western blots with the antibodies against active caspase 3, Bcl-x_L and β-actin. Representative results are shown. D: PAEC, control or treated with Ang II (10 μM) for 16 hours were used to prepare whole cell lysate. Equal amounts of protein lysates were immunoprecipitated with anti-Bax antibody followed by western blotting for Bcl-x_L or Bax. Densitometries are shown to the right showing the ratio of Bcl-x_L to Bax. All bar graphs show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Figure 5. Ang II induced apoptosis requires SHP phosphatase. PAEC were pretreated with the following inhibitors for 20 minutes prior to Ang II (10 μM) or AT2 agonist CGP-42112A (CGP, 10 μM) treatment for 16 hours. Neutral comet assays were performed and percentages of apoptotic cells were determined. A. PKC inhibitor chelerythrine

chloride (Chel, 1 μM) or sodium vanadate (Na_3VO_4, 20 μM), a broad spectrum tyrosine phosphatase inhibitor; B. PKC inhibitor Calphostin C (50 nM) C: SHP-1/2 phosphatase inhibitor (NSC-87877; 50 μM). Data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Figure 6. SHP-2 is activated by Ang II. A: PAEC were treated with 10 μM Ang II for the indicated times. Cell lysates were immunoprecipitated with anti-SHP-2. Immunoprecipitated SHP-2 protein was then subjected to phosphatase assays. B: SHP-2 phosphorylation in response to Ang II. PAEC were treated with 10 μM Ang II for the indicated times. Cell lysates were prepared and equal amounts of protein were immunoprecipated for SHP-2 and blotted for phospho-tyrosine. Panel to the bottom shows densitometry of phospho-SHP-2 bands. C: PAEC were transfected with wild-type (WT) SHP-2, dominant negative (DN) SHP-2, or the C459S-mutant (C459S-SHP-2) phosphatase inactive SHP-2. An expression vector for GFP-tagged H2B histone was co-transfected as a marker. Cells were untreated or treated with 10 μM Ang II for 24 hours and used for neutral comet assays. GFP-H2B was used to visualize transfected cells and propidium iodide was added to stain comet tails. D: Cells were treated ± SHP-1/2 phosphatase inhibitor (NSC-87877) prior to 10 μM Ang II or 10 μM AT2 agonist (CGP-42112A). Cell lysates were prepared and equal amounts of protein were used for western blotting for Bcl-x_L. Blots were stripped and probed for β-actin as a loading control. Lower panel shows densitometry analysis of Bcl-x_L normalized to β-actin. All

data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 7. Activation of SHP-2 and induction of apoptosis by Ang II require $G_{\alpha s}$ protein as an adaptor. A,B: PAEC were treated with $G_{\alpha s}$ inhibiting peptide or random peptide (0.5 μM) prior to treatment with Ang II (10 μM). A) PAEC lysates were prepared after 1 minute of Ang II exposure, and 100 μg of protein were used to immunoprecipitate SHP-2. Phosphatase assays were performed as described in the Methods. B) Neutral comet assays were performed, and the percentage of apoptotic cells were determined after Ang II treatment (10 μM, 24 hours).

Figure 8. Ang II destabilizes Bcl-x_L mRNA in PAEC. A: PAEC were untreated (-) or treated with Ang II (10 μM) for the indicated time. mRNA was prepared and q-PCR was performed to determine Bcl-x_L mRNA normalized to the internal GAPDH mRNA level. Results show mean +/- S.D, n = 4. * indicates $p < 0.05$. B: PAEC were treated with 10 μM Ang II for 8 hours, followed by treatment with 5 μg/ml actinomycin D for the indicated times. PCR was performed for Bcl-x_L. Open circles = controls; black circles = Ang II. Densitometry data was normalized to the zero time point for each condition. Data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. Experiment was repeated 3 times.

Figure 9. Ang II reduces nucleolin binding to the 3'UTR of the Bcl-x_L mRNA. A: Total RNA of untreated PAEC were extracted and PCR amplified for all three Bcl-x_L AU rich sites

and GAPDH as a control (Top left and right images). RNA-immunoprecipitation of nucleolin was also performed on the untreated cells (Bottom left). Cells were fixed with 4% paraformaldehyde, sonicated, and immunoprecipitated with anti-nucleolin antibody. RNA-protein complex was reverse-crosslinked for 2 hours, and proteinase K and DNase were added. Phenol/choroform RNA extraction was performed for cDNA synthesis. PCR was performed for all three AU-rich region of the 3' UTR of Bcl-x_L. B: After PAEC were treated with 10 μM Ang II for 16 hours, RNA-IP for nucleolin and IgG was done. PCR was performed for the second AU-rich region of the 3' UTR of Bcl-x_L and Bcl-2. Right panel shows densitometry of Bcl-x_L mRNA band. C: Cells were transfected with renilla-Bcl-x_L 3'UTR and co-transfected with a luciferase vector RSV-Luc construct to normalize for transfection efficiency. 6 hours after transfection, cells were placed in serum-free medium. Cells were treated with the SHP-1/2 phosphatase inhibitor (NSC-87877) prior to 10 μM Ang II exposure for 16 hours. Luciferase and renilla assays were performed on cell lysates. All data show means ± S.D. * Indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 1

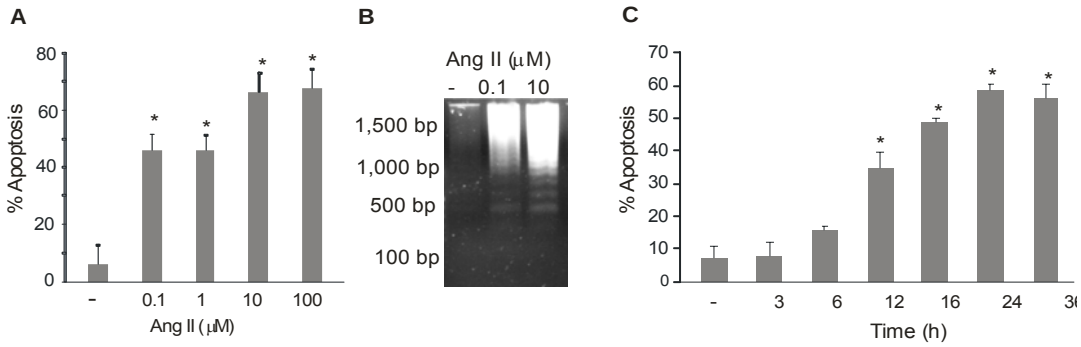

A
% Apoptosis
80
60
40
20
0
-
0.1
1
10
100
Ang II (μM)
B
Ang II (μM)
- 0.1 10
1,500 bp
1,000 bp
500 bp
100 bp
C
% Apoptosis
70
60
50
40
30
20
10
0
-
3
6
12
16
24
Time (h)

Figure 2

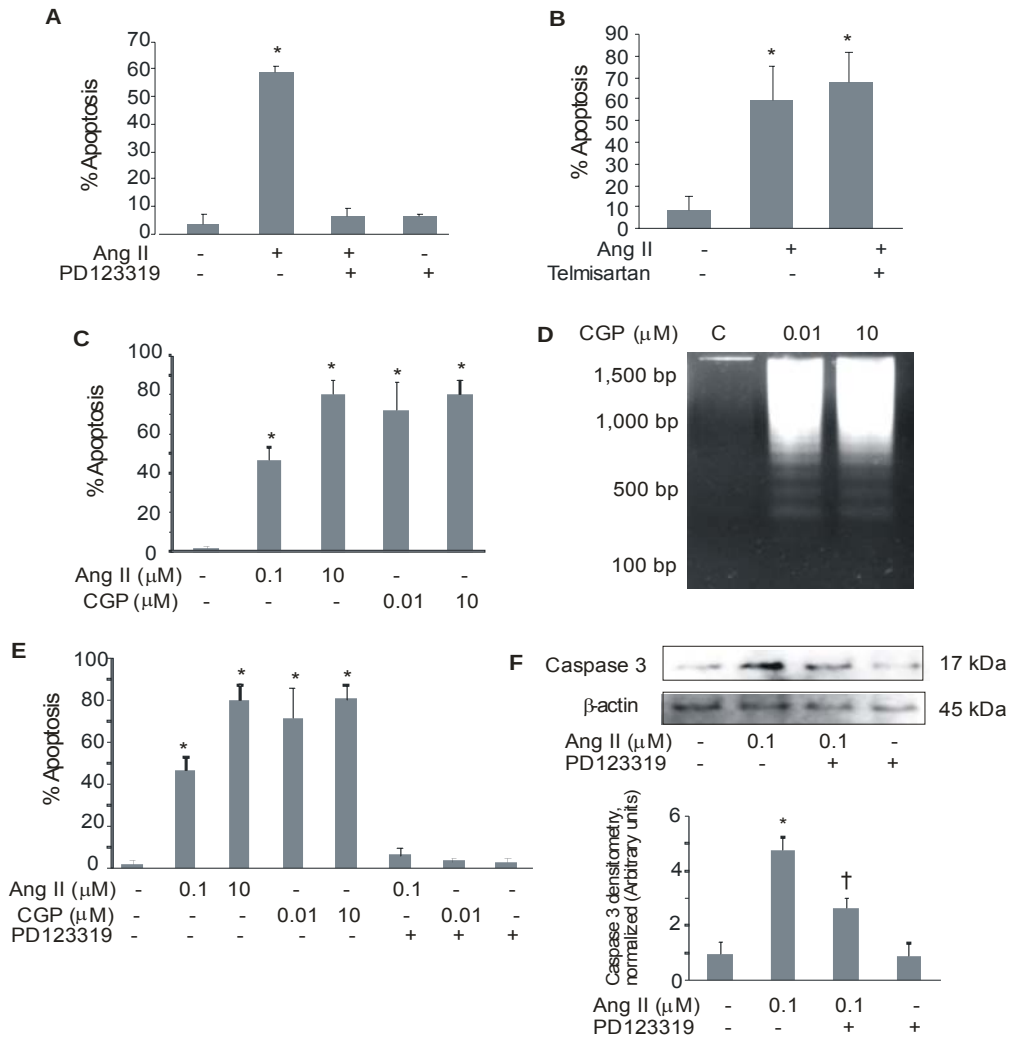

A
% Apoptosis
70
60
50
40
30
20
10
0
*
Ang II - + + -
PD123319 - - + +
B
% Apoptosis
90
80
70
60
50
40
30
20
10
0
* *
Ang II - + +
Telmisartan - - +
C
% Apoptosis
100
80
60
40
20
0
* * * *
Ang II (μM) - 0.1 10 - -
CGP (μM) - - - 0.01 10
D
CGP (μM) C 0.01 10
1,500 bp
1,000 bp
500 bp
100 bp
E
% Apoptosis
100
80
60
40
20
0
* * * *
Ang II (μM) - 0.1 10 - - 0.1 - -
CGP (μM) - - - 0.01 10 - 0.01 -
PD123319 - - - - - + + +
F
Caspase 3
17 kDa
β-actin
45 kDa
Ang II (μM) - 0.1 0.1 -
PD123319 - - + +
Caspase 3 densitometry, normalized (Arbitrary units)
6
4
2
0
* †
Ang II (μM) - 0.1 0.1 -
PD123319 - - + +

Figure 3

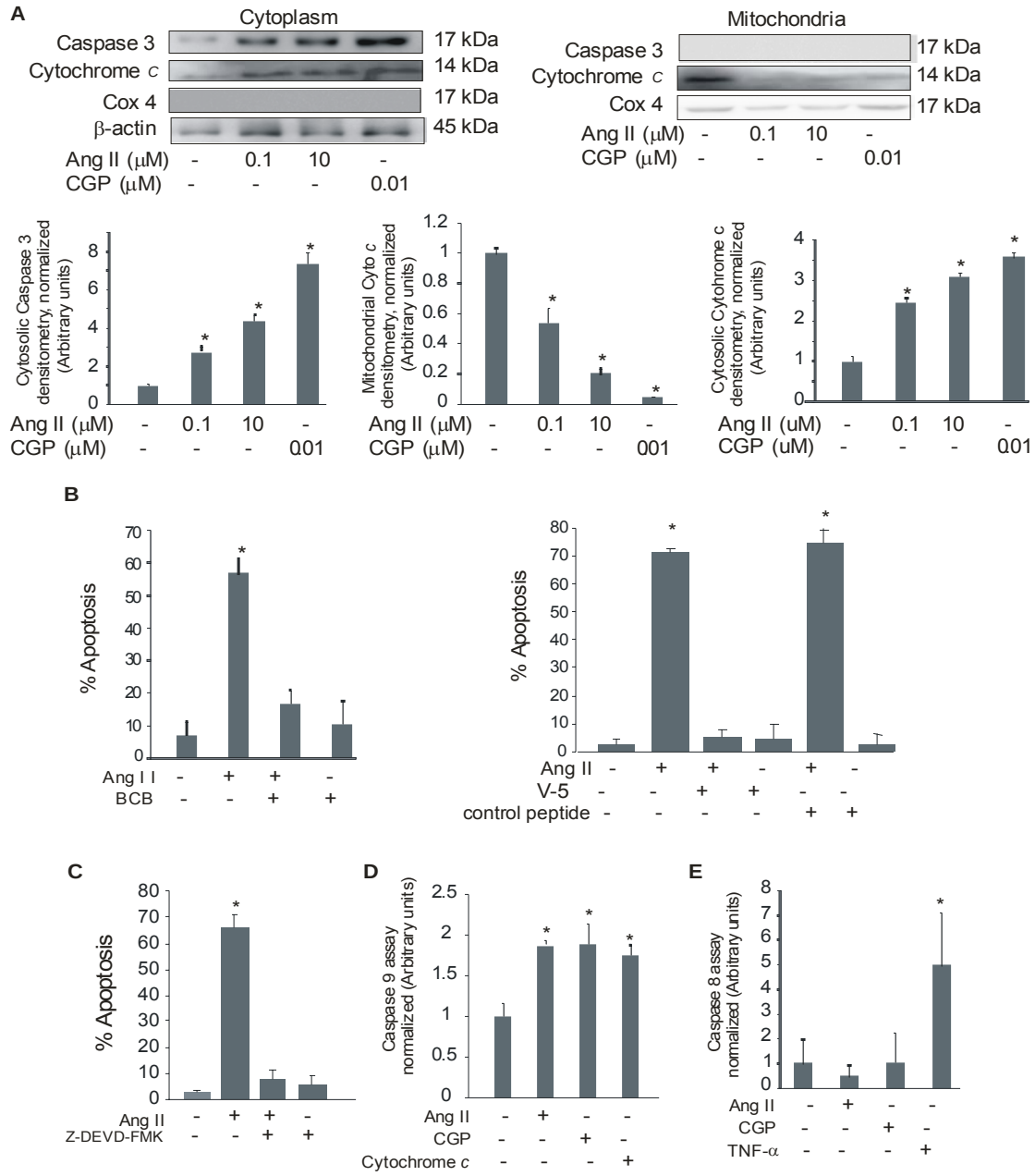

A
Cytoplasm
Caspase 3
Cytochrome c
Cox 4
β-actin
17 kDa
14 kDa
17 kDa
45 kDa
Ang II (μM) - 0.1 10 -
CGP (μM) - - - 0.01
Mitochondria
Caspase 3
Cytochrome c
Cox 4
17 kDa
14 kDa
17 kDa
Ang II (μM) - 0.1 10 -
CGP (μM) - - - 0.01
Cytosolic Caspase 3 densitometry, normalized (Arbitrary units)
Ang II (μM) - 0.1 10 -
CGP (μM) - - - 001
Mitochondrial Cyto c densitometry, normalized (Arbitrary units)
Ang II (μM) - 0.1 10 -
CGP (μM) - - - 001
Cytosolic Cytohrome c densitometry, normalized (Arbitrary units)
Ang II (uM) - 0.1 10 -
CGP (uM) - - - 001
B
% Apoptosis
Ang I I - + + -
BCB - - + +
% Apoptosis
Ang II - + + - + -
V-5 - - + + - -
control peptide - - - - + +
C
% Apoptosis
Ang II - + + -
Z-DEVD-FMK - - + +
D
Caspase 9 assay normalized (Arbitrary units)
Ang II - + - -
CGP - - + -
Cytochrome c - - - +
E
Caspase 8 assay normalized (Arbitrary units)
Ang II - + - -
CGP - - + -
TNF-α - - - +

Figure 4

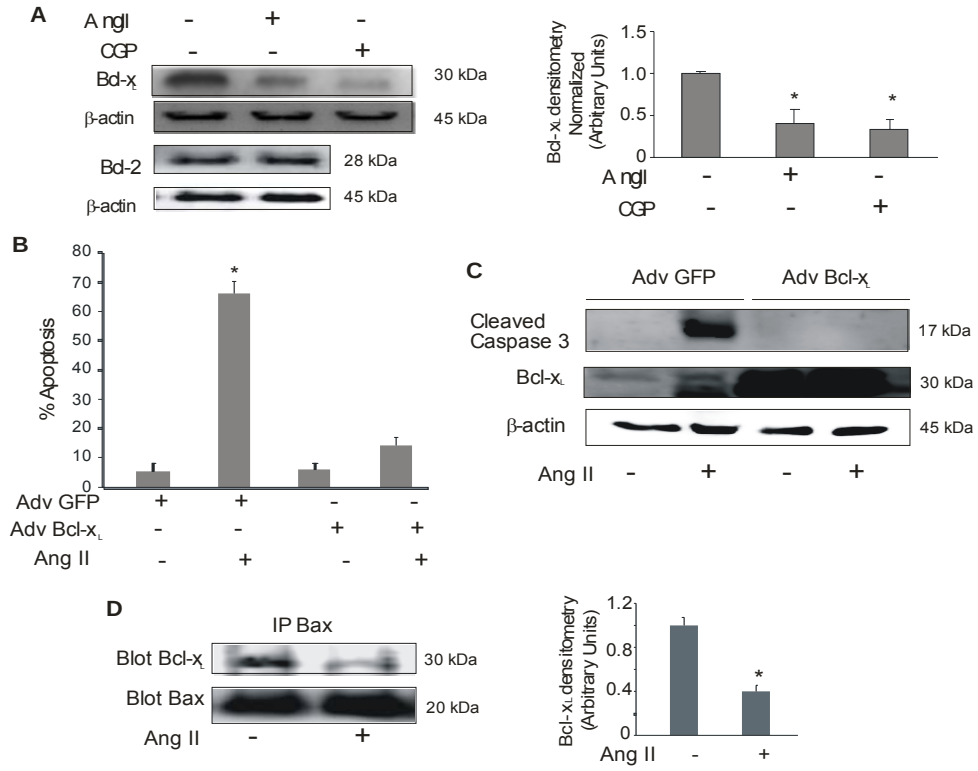

A
Angl - + -
CGP - - +
Bd-xL
β-actin
Bd-2
β-actin
30 kDa
45 kDa
28 kDa
45 kDa
Bcl-xL densitometry Normalized (Arbitrary Units)
1.5
1.0
0.5
0
Angl - + -
CGP - - +
B
% Apoptosis
80
70
60
50
40
30
20
10
0
Adv GFP + + - -
Adv Bcl-xL - - + +
Ang II - + - +
C
Adv GFP
Adv Bcl-xL
Cleaved Caspase 3
Bcl-xL
β-actin
17 kDa
30 kDa
45 kDa
Ang II - + - +
D
IP Bax
Blot Bcl-xL
Blot Bax
30 kDa
20 kDa
Ang II - +
Bcl-xL densitometry (Arbitrary Units)
1.2
0.8
0.4
0
Ang II - +

Figure 5

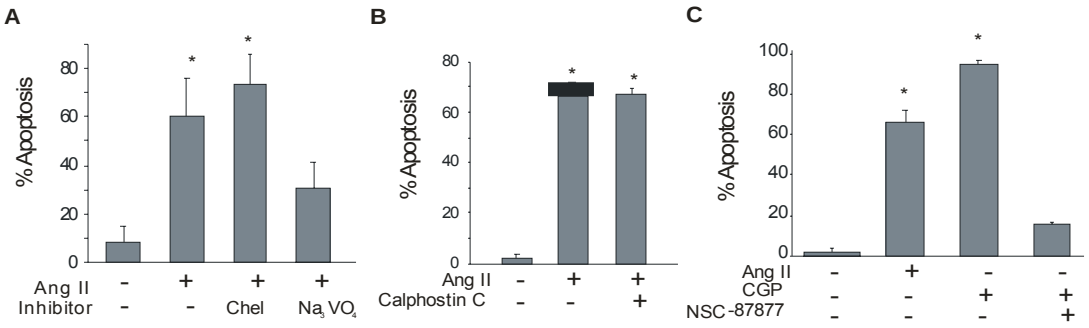

A
% Apoptosis
0
20
40
60
80
*
*
Ang II - + + +
Inhibitor - - Chel Na_3VO_4
B
% Apoptosis
0
20
40
60
80
*
*
Ang II - + +
Calphostin C - - +
C
% Apoptosis
0
20
40
60
80
100
*
*
Ang II - + - -
CGP - - + +
NSC-87877 - - - +

Figure 6

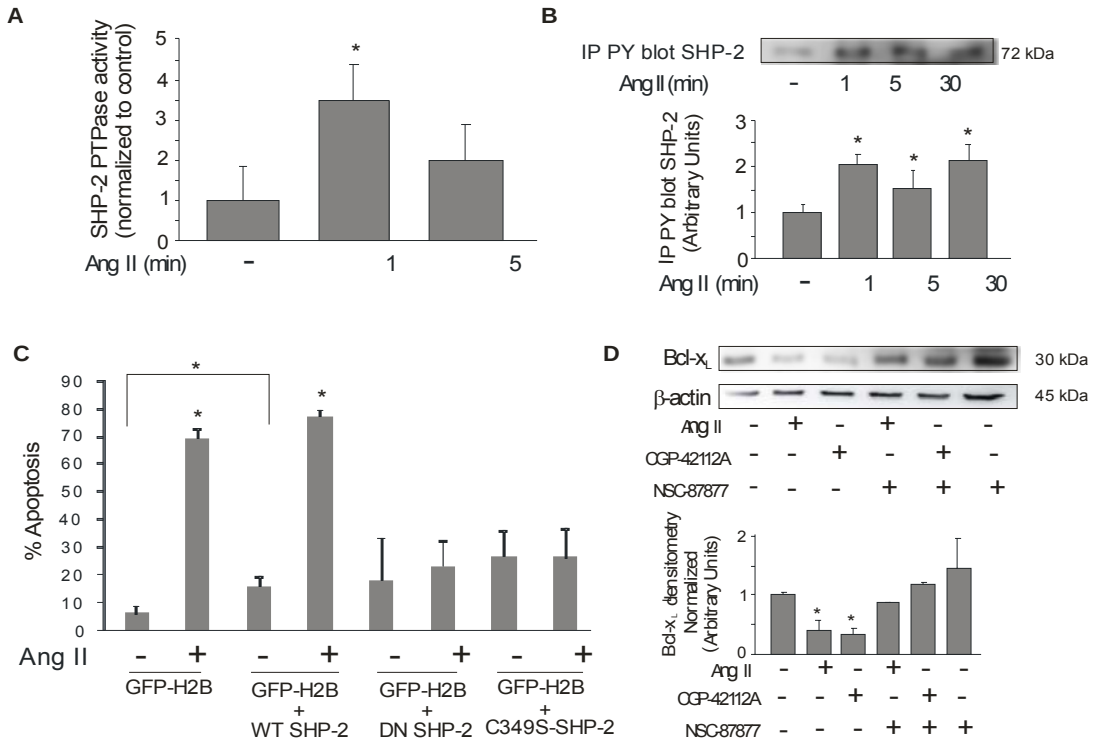

A
SHP-2 PTPase activity (normalized to control)
Ang II (min)
B
IP PY blot SHP-2
72 kDa
Ang II (min)
IP PY blot SHP-2 (Arbitrary Units)
C
% Apoptosis
Ang II
GFP-H2B
GFP-H2B + WT SHP-2
GFP-H2B + DN SHP-2
GFP-H2B + C349S-SHP-2
D
Bcl-xL
30 kDa
β-actin
45 kDa
Ang II
CGP-42112A
NSC-87877
Bcl-xL densitometry Normalized (Arbitrary Units)

Figure 7

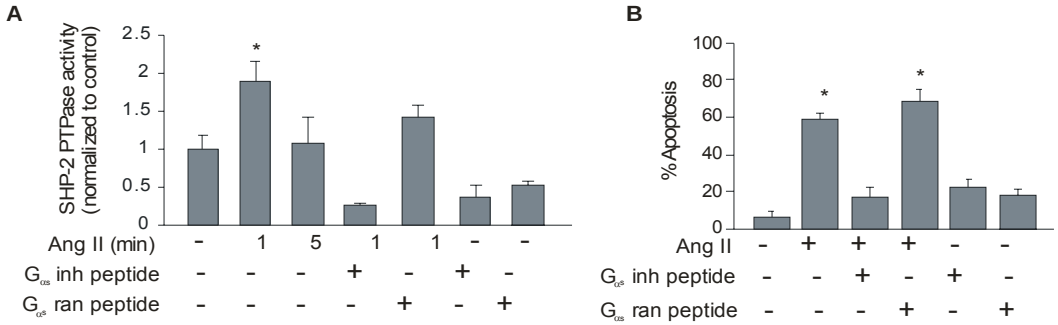

A
SHP-2 PTPase activity (normalized to control)
2.5
2
1.5
1
0.5
0
*
Ang II (min) - 1 5 1 1 - -
Gαs inh peptide - - - + - + -
Gαs ran peptide - - - - + - +
B
% Apoptosis
100
80
60
40
20
0
*
*
Ang II - + + + - -
Gαs inh peptide - - + - + -
Gαs ran peptide - - - + - +

Figure 8

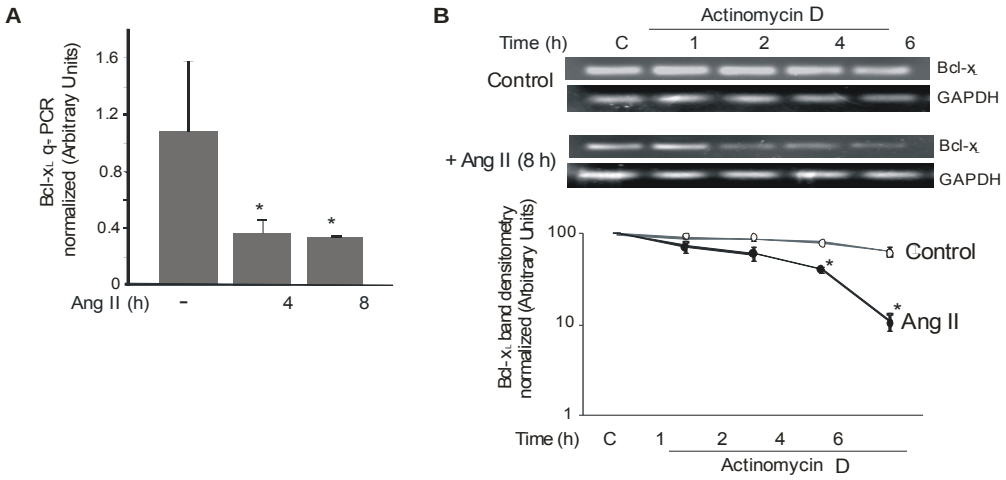
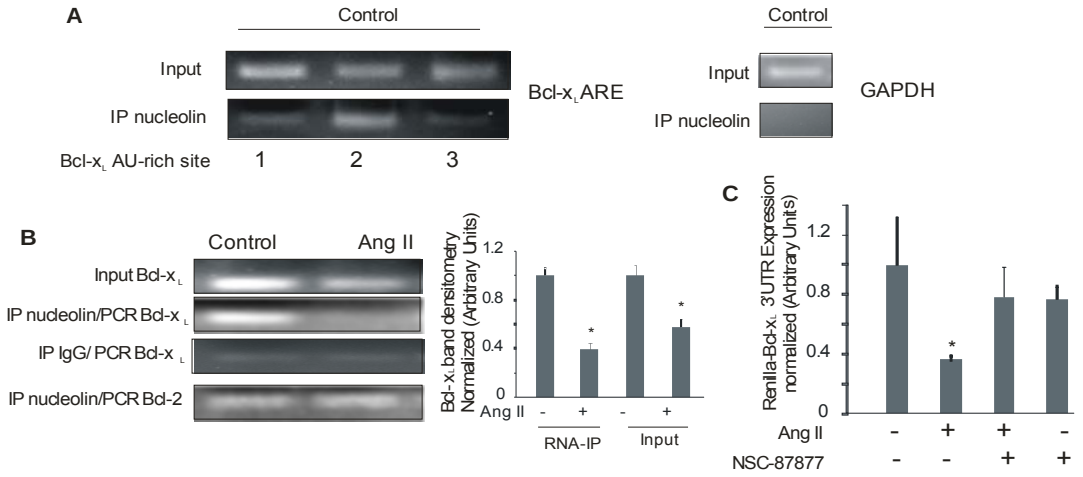
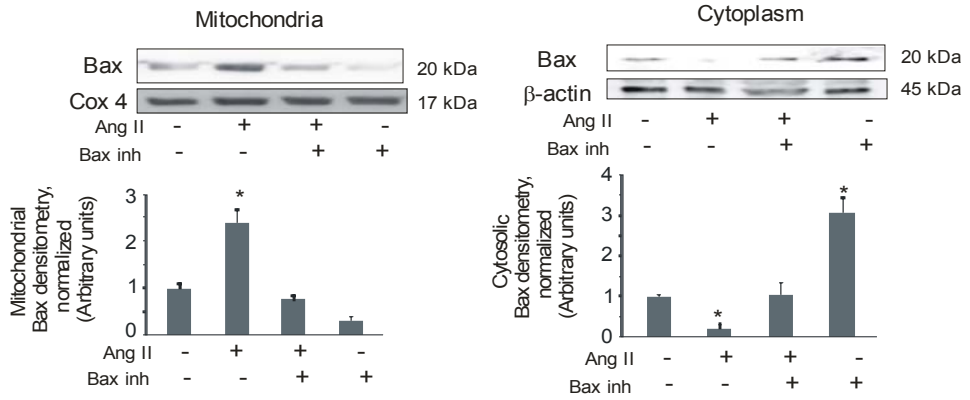

A
Bcl-xL q-PCR normalized (Arbitrary Units)
1.6
1.2
0.8
0.4
0
Ang II (h) -
4
8
B
Actinomycin D
Time (h) C 1 2 4 6
Control
Bcl-x
GAPDH
+ Ang II (8 h)
Bcl-x
GAPDH
Bcl-x band densitometry normalized (Arbitrary Units)
100
10
1
Control
Ang II
Time (h) C 1 2 4 6
Actinomycin D

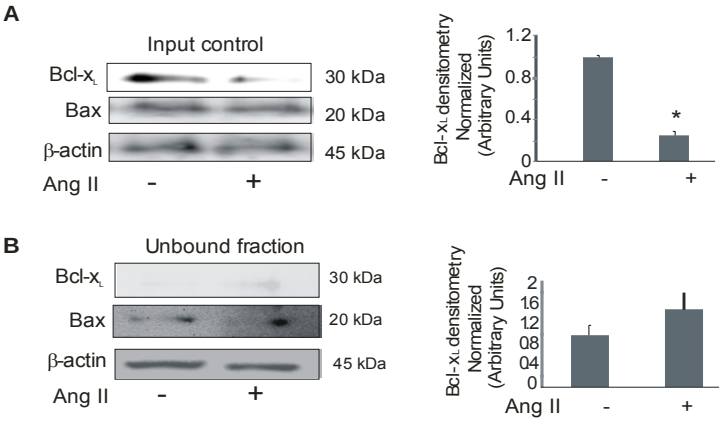
Figure 9


A
Control
Input
IP nucleolin
Bcl-x$_L$ AU-rich site
1
2
3
Bcl-x$_L$ ARE
Control
Input
IP nucleolin
GAPDH
B
Control
Ang II
Input Bcl-x$_L$
IP nucleolin/PCR Bcl-x$_L$
IP IgG/ PCR Bcl-x$_L$
IP nucleolin/PCR Bcl-2
Bcl-x$_L$ band densitometry Normalized (Arbitrary Units)
1.2
0.8
0.4
0
Ang II
RNA-IP
Input
C
Renilla-Bcl-x$_L$ 3'UTR Expression normalized (Arbitrary Units)
1.2
0.8
0.4
0
Ang II
NSC-87877

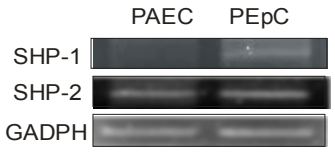
Supplementary Figure 1



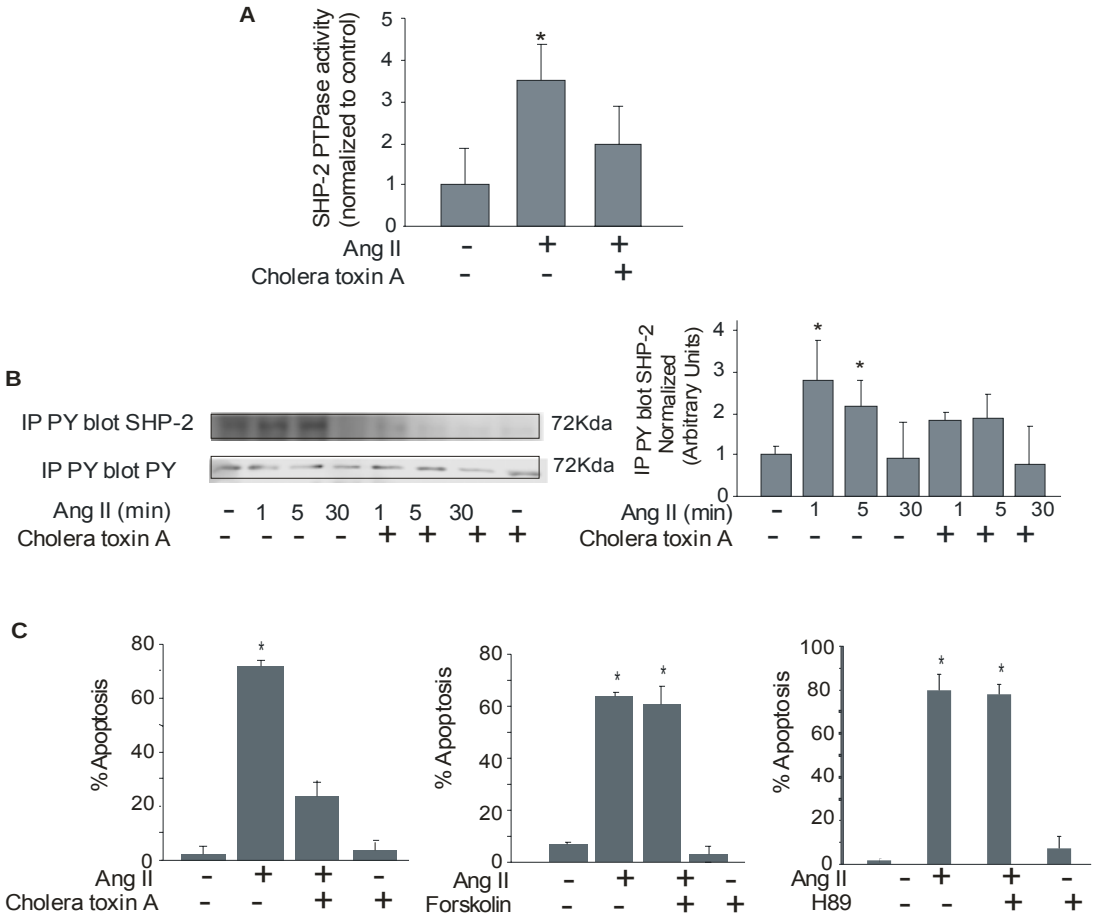
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplemental Section:

Figure S1: PAEC were treated for 20 minutes with either the Bax inhibitory peptide V5 (100 μM) or control random peptide (100 μM). Ang II (10 μM) was then added for 16 hours. Mitochondria-free cell lysates (cytoplasm) or mitochondrial extracts were prepared and western blotted for Bax protein; blots were stripped and reprobed for β-actin (loading control for cytoplasm) or for COX4 (loading control for mitochondria). Densitometry results normalized to β-actin or COX4 are shown in lower panels. All bar graphs show means ± SD. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Figure S2. Ang II affects Bax/Bcl-x_L association. PAEC, control or treated with Ang II (10 μM) for 16 hours were used to prepare cell lysate. A. Input: western blots of endogenous Bcl-x_L and Bax in whole cell lysate; blot was stripped and reprobed for β-actin. B. Unbound fraction: after immunoprecipitation of Bax (shown in Figure 4D), the supernatant containing the unbound proteins were used for western blotting for Bcl-x_L and Bax; blot was stripped and reprobed for β-actin. Densitometries are shown to the right of each group of gels showing the ratio of Bcl-x_L to Bax; for the input and unbound fraction, the ratio is normalized to β-actin. All bar graphs show means ± SD. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Figure S3: SHP-2 is expressed in PAEC. RT-PCR analysis of SHP-1 and SHP-2 gene expression in pulmonary epithelial cells (right panel) and PAEC (left panels). RT-PCR of GAPDH is shown as a control.

Figure S4: Activation of SHP-2 and induction of apoptosis by Ang II require inactive $G_{\alpha s}$ protein but PKA. A. Phosphatase assays were performed on cell lysates prepared after 1 minute of Ang II exposure. B. SHP-2 tyrosine phosphorylation was determined in cell lysates following the indicated times of Ang II treatment. SHP-2 protein was immunoprecipitated and used for blotting with an anti-phosphotyrosine antibody. Band densitomery analysis is shown to the right. C. Neutral comet assays were performed on PAEC pretreated with cholera toxin A (100 nM), forskolin (50 μM) or H89 (10 μM) prior to treatment of Ang II (10 μM; 24 hours). Percentage of apoptotic cells were determined. All bar graphs show means ± SD. *Indicates statistical significance from control, $p < 0.05$, n = 3. All experiments were repeated at least 3 times.

Hepatocyte Growth Factor Abrogates Angiotensin II-induced Apoptosis in Primary Lung Endothelial Cells

Young H. Lee, Ana P. Marquez, Ognoon Mungunsukh and Regina M. Day

Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD, 20814

Correspondences to: Regina M. Day, PhD, Department of Pharmacology, C2023, 4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA. Fax: 301-295-3220. E-mail: rday@usuhs.mil

Running head: HGF Inhibits Angiotensin II-induced Intrinsic Apoptosis

Key words: intrinsic apoptosis, mRNA half-life, Bcl-x_L, nucleolin, Angiotensin II, Hepatocyte Growth Factor

Abbreviations: FBS, fetal bovine serum; HGF, Hepatocyte Growth Factor; Ang II, Angiotensin II; BSA, bovine serum albumin; UTR, Untranslated Region

Summary:

Pulmonary fibrosis is characterized by the loss of lung epithelial and endothelial cells, the proliferation of myofibroblasts, and alteration of the lung extracellular matrix. Hepatocyte growth factor (HGF), an endogenous tissue repair factor, has been demonstrated to attenuate lung fibrosis in murine models, but the mechanism is not yet understood. We hypothesized that HGF may block epithelial and endothelial cell apoptosis induced by profibrotic factors. Our laboratory recently demonstrated that Ang II activates the intrinsic apoptotic pathway in primary lung endothelial cells by reducing the level of the anti-apoptotic protein Bcl-x_L. We determined that Ang II decreased the Bcl-x_L mRNA half-life by reducing its binding to nucleolin, a protein that normally binds to a 3' AU-rich region and stabilizes the Bcl-x_L mRNA. We utilized primary cultures of pulmonary artery endothelial cells (PAEC) and *ex vivo* cultures of rat lung tissue to investigate HGF inhibition of Ang II-induced apoptosis. Co-administration of HGF abrogated Ang II-induced activation of caspase 3 and the release of LDH in rat lung tissue explants. When PAEC were treated with HGF, apoptosis by Ang II was completely abrogated, as determined by the neutral comet assay. HGF inhibited Ang II-induced cytochrome *c* release, caspase 3 activation, and DNA fragmentation. HGF prevented Ang II-induced decrease of Bcl-x_L protein. RNA-IP experiments demonstrated that HGF stabilized Bcl-x_L mRNA by increasing nucleolin binding to the 3'UTR region. We found that Ang II caused nuclear translocation of nucleolin. Western blots demonstrated that HGF-stabilized Bcl-x_L mRNA was associated with cytoplasmic localization of nucleolin. Nucleolin localization to the cytoplasm and cell survival required Erk1/2 activation by HGF.

Our data indicate that HGF inhibits Ang II-induced apoptosis by stabilizing Bcl-x_L mRNA via ERK1/2 phosphorylation of nucleolin.

Introduction:

Hepatocyte growth factor (HGF) was originally identified as a potent mitogen for mature hepatocytes but has shown the ability to exert potent motogenic, mitogentic, and motogenic activities on a wide variety of cells via activation of the tyrosine kinase receptor, Met (1).

Studies show that HGF functions in a variety of organs as a tissue repair factor. HGF, an anti-apoptotic factor, has been shown to promote normal repair and prevent aberrant repair mechanisms that can lead to fibrotic remodeling in several organ systems, including the lung, heart, kidney and liver (2-5). Animal studies show that administration of HGF induces proliferation of epithelial and endothelial cells and promotes normal tissue regeneration while at the same time preventing the development of fibrosis. The simultaneous or delayed administration of HGF to mice with bleomycin-induced lung injury prevents both endothelial and epithelial cell apoptosis, the appearance of fibroblast foci, and the accumulation of collagen found in pulmonary fibrosis (2,6). Administration of adenovirally expressed HGF or HGF gene expression using *in vivo* transient plasmid transfection prevented bleomycin-induced increased collagen expression in the lung; these experiments also showed that HGF preserved normal lung architecture (7,8). HGF binding to its receptor Met activates a number of signaling in a cell type-dependent manner. Previous studies reported that HGF activates the Erk1/2 and the PI3K/Akt pathways, major signal transduction pathways associated with cellular growth and the

prevention of apoptosis (9,10). In pulmonary artery endothelial cells, HGF activation of PI3K pathway was shown to upregulate Bcl-x_L mRNA.

The vasoactive peptide angiotensin II (Ang II) was first studied for its role in blood pressure homeostasis. However, Ang II has been shown to play a key role in the progression of lung, liver, kidney and heart fibrosis (11,12). Ang II has been shown to induce the growth and transdifferentiation of myofibroblasts as well as the apoptosis of epithelial and endothelial cells (13,14). Local synthesis of Ang II has been observed in fibrotic plaques, and human lung myofibroblasts from patients with idiopathic pulmonary fibrosis were found to generate Ang II (15). Furthermore, the inhibition of Ang II-induced fibrosis was associated with the reduction of epithelial and endothelial cell apoptosis (16). Ang II was found to induce apoptosis via the intrinsic apoptotic pathway in lung epithelial cells (17). Our laboratory showed that ang II also induced intrinsic apoptosis in PAEC. Ang II-induced release of cytochrome *c,* activation of caspase 3, and DNA laddering. We found that Ang II treatment of PAEC resulted in the loss of Bcl-x_L protein and destabilization of its mRNA. The stabilization of Bcl-x_L mRNA is dependent upon its 3'UTR, prominently at an AU-rich element (ARE), being bound to nucleolin. Nucleolin is a 110 kDa multifunctional protein, ubiquitously present in the nucleus, cytoplasm, and in the cell membrane (18,19). Phosphorylated nucleolin resides in the cytoplasm, while the dephosphorylated nucleolin accumulates in the nucleus (20). We found that Ang II causes nuclear translocation of nucleolin prevented binding to the second AU rich region of the Bcl-x_L mRNA 3'UTR. The absence of the cytoplasmic nucleolin caused mRNA instability and degradation.

Here, we examined the mechanism of HGF-mediated inhibition of Ang II-induced apoptosis in primary cultures of PAEC. HGF inhibited Ang II-induced release of cytochrome *c* and activation of caspase 3. HGF inhibited the nuclear translocation of nucleolin, resulting in increased nucleolin in the cytoplasm to protect the Bcl-x_L mRNA from Ang II-induced degradation. HGF activated Erk1/2 to phosphorylate nucleolin, thereby inhibiting its nuclear translocation. Together, these data suggest that HGF protects endothelial cells against Ang II-induced apoptosis via activated Erk1/2-mediated cytoplasmic localization of nucleolin which stabilizes Bcl-x_L mRNA, thus inhibiting the intrinsic apoptotic pathway.

Experimental Procedures:

Reagents- Angiotensin II was purchased from Bachem, Inc. (Torrance, CA). Antibodies against cytochrome *c* and β-Actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-cleaved caspase 3 was purchased from Cell Signaling Technology (Danvers, MA). The Renilla-Bcl-x_L construct was the gift of Dr. Tim Bowden. HGF was the gift of Dr. GF Vande Woude (Van Andel Research Institute, Grand Rapids, MI).

Cell Culture- Bovine pulmonary artery endothelial cells (PAEC) were purchased from American Type Culture Collection (Manassas, VA). Passage 2-8 cells were used for all experiments and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin and 0.5% fungizone (Invitrogen). Cells were grown in 5% CO_2 at 37^oC in a humidified atmosphere in a culture incubator.

Cell Lysate- Cells were washed with ice-cold PBS and lysed in 50 mM Hepes, pH 7.4; 1% (v/v) Triton X-100; 4 mM EDTA; 1 mM sodium fluoride; 0.1 mM sodium orthovanadate; 1 mM tetrasodium pyrophosphate; 2 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin; and 10 μg/ml aprotinin. Lysates were incubated 15 minutes on ice, vortexed, and insoluble materials were removed by centrifugation (14,000 × g, 10 minutes, 4°C). For immunoprecipitation of Bax, cells were washed with cold PBS and lysed in CHAPS buffer (50 mM Tris-HCl; 1mM EGTA; 1% (w/v) CHAPS; 10% glycerol; 50 mM sodium fluoride; 1 mM sodium orthovanadate; 2 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin; and 10 μg/ml aprotinin). Equal concentrations of protein from cell lysates were incubated with primary antibody (1:1000 dilution). GammaBind Plus beads (1:100 dilution; Amersham Biosciences, Piscataway, NJ) were added and samples were rotated at 4°C overnight. The beads were centrifuged at 10,000 × g for 10 minutes at 4°C, washed twice with lysis buffer containing protease and phosphatase inhibitors. To elute, beads were resuspended in 25 μl Laemmli buffer incubated for 5 minutes at 95°C.

Western blots- Whole cell lysates (10 μg of total protein) were subjected to SDS polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. Membranes were blocked with 5% BSA in Tween 20 Tris Buffered Saline (TTBS, Tris Buffered Saline, 0.1% Tween 20) for 1 hour at ambient temperature. Membranes were then incubated overnight at 4°C with primary antibody (1:1000 dilution) in TTBS containing 0.5% (w/v) BSA. Membranes were washed three times with TTBS for 10 minutes, followed by incubation with horse-radish peroxidase-labeled secondary antibody (1:1000 in TTBS) for 1 hour at ambient temperature. For

protein detection, membranes were washed for 3 hours with TBS, incubated in ECL (Pierce Biotechnology), and analyzed on a FujiFilm Image Reader LAS-1000Pro (FujiFilm USA Inc., Valhalla, NY).

Mitochondria and mitochondria-free cytosolic protein extraction – Mitochondria extraction was performed using the Mitochondria Isolation Kit for Cultured Cells according to the manufacturer's protocol (Pierce, Rockford, IL).

Neutral Comet Assay- The neutral comet assay was used to measure double stranded DNA breaks as an indication of apoptosis (21). After treatment, cells were embedded in 1% (w/v) low-melting agarose and placed on comet slides (Trevigen, Gaithersburg, MD). Slides were placed in lysis solution (2.5 M NaCl, 1% Na-lauryl sarcosinate, 100 mM EDTA, 10 mM Tris base, 0.01% Triton X-100) for 30 minutes, followed by a wash in 1 × TBE buffer (0.089 M Tris; 0.089 M Boric acid; and 0.002 M EDTA, pH 8.0). Nuclei were electrophoresed for 10 minutes at 18 V in 1×TBE. Cells were then fixed with 75% ethanol for 10 minutes and air-dried overnight, stained with 1×Sybr® Green (Molecular Probes, Eugene, OR) or propidium iodide (Sigma-Aldrich, St. Louis, MO) and visualized with an Olympus FV500 confocal laser scanning microscope (Olympus Imaging America, Center Valley, PA) using 20 × magnification at 478 nm excitation, 507 nm emission wavelengths for Sybr Green and at 535 nm excitation, 617 nm emission wavelengths for propidium iodide. All cells were counted in randomly selected fields for each treatment group and assigned into

type A, B or C comet categories, based on their tail moments. Type C comets were defined as apoptotic cells (22).

DNA laddering assay- Cells were harvested in the medium and pelleted at 1000 × g. Pellets were resuspended and incubated on ice in lysis buffer (1× Tris-EDTA, 0.2% Triton at pH 8.0) for 15 minutes. Resuspended pellet was centrifuged (14,000 × g, 10 minutes at 4^{o}C) and supernatant containing the fragmented DNA was collected. RNase A (final concentration at 60mg/ml) was added and incubated for 30 minutes at 37^{o}C. SDS was added to a final concentration of 0.5% along with 150 μg/ml of proteinase K and incubated 2 hours at 50^{o}C. 0.1 volume of 5 M NaCl and 1 volume of ice cold isopropanol was added and samples were incubated on ice for 10 minutes. The samples were centrifuged at 13,000 × g for 15 minutes at 4°C. The DNA pellet was briefly dried and dissolved in 20ul of TE buffer, followed by electrophoresis (~2 hours, 20V) in 1.5% agarose.

RNA isolation and reverse transcription (RT)- Total RNA was obtained from bovine PAEC using the RNeasy kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-Free DNAse Set (Qiagen). RNA concentrations were determined spectroscopically at 260 nm (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE). RNA (1.0 μg) was subjected to RT with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Semi-quantitative RT-PCR- 1μl of cDNA from the RT reaction was used for PCR reaction containing 0.4 μM each forward and reverse primer, 200 μM each

dNTP, 1 U of i*Taq* DNA polymerase, and 1× PCR buffer (BioRad Laboratories Inc., Hercules, CA). The PCR primers for Bcl-x_L mRNA were: 5′-GGT ATT GGT GAG TCG GAT CG and 5′-GCT GCA TTG TTC CCG TAG AG. PCR reactions were optimized for annealing temperatures using a temperature gradient in a BioRad iCycler. Reactions were carried out for 25 cycles using the following conditions: 95°C 1 minute; 60°C 45 seconds; 72°C 1.5 minutes. The last cycle extension was for 10 minutes at 65°C. PCR primers for GAPDH were: 5'- GAA GCT CGT CAT CAA TGG AAA and 5'- CCA CTT GAT GTT GGC AGG AT. PCR reactions were analyzed on a 1.5% agarose gel in Tris EDTA buffer and bands were visualized using ethidium bromide.

Determination of mRNA Half-life- Bovine PAEC were treated as indicated, then incubated with 5 μg/ml actinomycin D for time courses between 30 minutes and 8 hours. Total RNA was isolated and the level of Bcl-x_L mRNA was determined by qPCR or by semi-quantitative RT-PCR (primers listed above). Levels of GAPDH mRNA were determined by gel electrophoresis and used for normalization of the semi-quantitative RT-PCR (primers are listed above). ImageJ software (http://www.uhnresearch.ca/facilities/wcif/index.htm) was used for quantitation.

Protein stability assay- To measure the rate of degradation of Bcl-x_L and nucleolin, cells were treated with 50 μg/ml of cycloheximide 16 hours after the Ang II treatment to prevent further protein synthesis. The cell lysates were prepared from samples taken at different time points and the western blotting was used to determine protein levels.

Plasmid transfection - One day prior to transfection, cells were plated at 1.4×10^5 cells per well in a 12-well plate. DNA (1 μg) per well was transfected using the FuGENE 6 Transfection Reagent (Roche Applied Science, San Francisco, CA), according to the manufacturer's instructions, in serum-free, antibiotic-free medium. For luciferase assays, transfection mixtures contained a ratio of renilla-Bcl-x_L reporter construct to luciferase control vector (RSV-Luc) of 6:1 to normalize transfection efficiency.

Dual luciferase assay- Transfected cells were washed twice with cold PBS, lysed with passive lysis buffer, and assayed for firefly and Renilla luciferase activities using the Dual Luciferase Assay (Promega, Madison, WI) according to the manufacturer's instructions in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA-IP- Cells were harvested by centrifugation at 1000 x g for 3 minutes and resuspended in 10 ml of PBS. RNA and protein complexes were cross-linked by adding of 1.0% (v/v, final concentration) of formaldehyde and incubation at room temperature for 10 minutes with gentle mixing. Cross-linking was quenched by addition of glycine (pH 7.0, 0.125 mol/L final concentration), at room temperature for 5 minutes. Cells were washed twice with 10 ml PBS containing protease and RNAse inhibitors. The pellet was resuspended in 0.2 ml NP-40 buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40, protease inhibitors, RNase inhibitor) and incubated on ice for 10 minutes. Nuclei were pelleted by centrifugation at 1400 x g for 5 minutes at 4^oC. Supernatant was sonicated three times for 20 seconds each at output level 6 of a

sonic dismembrator, Model 100 (Fisher Scientific, Pittsburg, PA). The samples were cleared by centrifugation for 10 minutes, 14000 rpm at 4°C, and diluted 10 fold into IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, protease inhibitors, RNase inhibitor) to a final volume of 1 ml per immunoprecipitation reaction. A 1% aliquot was preserved as an input sample. Nucleolin antibody (1:1000 dilution) was added to each tube and immune complexes were allowed to form by gentle mixing on a rotating platform at 4°C overnight. To collect immune complexes, 50 μL of Sepharose Beads were added and mixed gently for 2 hours followed by centrifugation at 60 x g for 2 minutes at 4°C. The immune complexes were washed for 5 minutes each with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl (0.25M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and twice with TE buffer. Each wash was followed by 60 x g centrifugation for 1 minute. Immune complexes were eluted in 500 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$, RNase inhibitor). NaCl was added to a final concentration of 200 mM then samples were placed at 65°C for 2 hours to reverse crosslinking. Next, 20 μl of 1 M Tris-Cl pH 6.5, 10 μl of 0.5 M EDTA, and 20 μg of Proteinase K were added to each sample and incubated at 42°C for 45 minutes. RNA was extracted using phenol:chloroform with glycogen as a DNA-carrier. DNA was removed with DNase (Qiagen). RT-PCR was performed using 1μl of the cDNA reaction for 25 cycles: denaturing was performed at 95°C for 1 minute, annealing for 45 second at 60°C, and polymerase reaction for 1.5 minute at 72°C. Primers for Bcl-x_L ARE-2 were 5'-ACC TTC CTC

AAT TGT CGT GG-3' and 5'-GGG GAA AAG GGT CAG AAA C-3' and Bcl-2 ARE were 5'- TGC TTT TGA GGA GGG CTG CAC and 5'- ACT GCC TGC CAC AGA CCA GC.

Ex-vivo Lung Explants- All treatment of animals was according to National Institutes of Health, Department of Defense, and institutional guidelines. Sprague-Dawley rats (Taconic, Germantown, NY) were housed 1 female with litter per cage in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at 21 ± 2^{o}C, 50% ± 10% humidity, and 12-h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) and water were freely available. Rat pups were obtained on postnatal day 10-11. Animals were anesthetized with pentobarbital and decapitated, or euthanized with Fatal-Plus. The surface of the anterior chest wall and upper abdomen were sterilized with 70% ethanol. After the trachea was exposed, a small nick was made to insert a 22 gauge needle with a short piece of polyethelene tube attached. Through a midline abdominal incision the chest cavity was exposed and the animal was exsanguinated by severing the abdominal aorta. The right ventricle was punctured and the lungs were perfused with sterile PBS to remove the blood. The trachea, lungs, and the heart were then aseptically dissected from the animal.

To obtain lung slices for the *ex-vivo* culture, the lungs were inflated with 1% low melting point agarose dissolved in RPMI medium. The agarose was instilled into the trachea using a syringe and fully inflated the lungs. The lungs were placed in a sterile cell culture plates and at 4°C for at least 30 minutes to solidify the agarose. The heart was then excised from the lung and each lobe of the lung was embedded on a cutting board with 1%

agarose to prevent movement. The agarose-filled and embedded lungs were then chopped on a MclLwain tissue chopper (GeneQ Inc. Quebec, Canada) into 500 μm thick slices. The lung explant slices were incubated in RPMI containing 10% FBS, 1% penicillin/streptomycin and 0.5% fungizone for an hour in 37°C in a humidified chamber with 5% CO_2. Lung slices were transferred to a 24-well cell culture plate with treatment for 16 to 24 hours in 37°C humidified chamber with 5% CO_2.

For immunohistochemistry and microsopcy, the agarose inflated lungs were fixed by immersion in 10% buffered neutral formalin for 24-48 hours. The fixed lungs were embedded in Tissue-Tek OCT compound (VWR) and stored at -20ºC. Lung slices of 10 to 20 μm thickness were prepared using a SL-3000 Edge Cryostat (HACKER Instruments & Industries Inc, Winnsboro, SC). The slices were then processed using standard histological techniques. Primary antibodies of cleaved caspase 3 (Cell Signaling Technology, Beverly MA) were used in final dilution of 1:200. Negative controls were done by omitting the respective primary antibodies.

Statistical Analysis- Means ± standard deviations (SD) were calculated, and statistically significant differences between two groups were determined using the Student's *t* test. For three or more groups, statistical analysis was performed using one-way ANOVA, followed by the Bonferroni post-analysis, as appropriate; $p < 0.05$ was considered statistically significant. For mRNA half-life, linear regression was calculated and confidence intervals determined. Statistical software for all analysis was SigmaStat 3.1 (Point Richmond, CA).

RESULTS

HGF inhibits Ang II-induced apoptosis. HGF has been demonstrated to attenuate cellular apoptosis and reverse fibrosis in the lung (2,6). Previously, we investigated the effects of Ang II on primary pulmonary artery endothelial cells (PAEC) and found that 10 μM Ang II caused DNA fragmentation in 24 hours (Lee *et al.* 2010. In press). The effect of HGF on Ang II-induced apoptosis in PAEC was examined using the neutral comet assay, which detects chromosomal breakdown as a function of apoptosis. Pretreatment with HGF significantly inhibited the Ang II-induced apoptosis (Fig 1A).

HGF inhibits Ang II-induced intrinsic apoptosis. Previously, we showed that Ang II activates the intrinsic pathway, including cytochrome *c* release, caspase 9 and caspase 3 activation and DNA laddering. To determine whether HGF can inhibit Ang II-induced intrinsic apoptosis, cells were pretreated with HGF prior to Ang II treatment. Western blot analysis revealed significant inhibition of Ang II-induced caspase 3 activation and the release of cytochrome *c* with the HGF treatment (Fig 2A). To confirm that the cells are indeed undergoing apoptosis, we performed DNA laddering, where 25 ng/ml HGF pretreatment inhibited Ang II-induced DNA fragmentation (Fig 2B).

HGF inhibits Ang II- reduced Bcl-x_L mRNA half-life. We recently reported that Ang II reduced Bcl-x_L protein levels within 16 hours of treatment (Lee *et al.* 2010, in press). However, pretreatment with HGF blocked Ang II-induced

downregulation of Bcl-x_L (Fig 3A). The data was confirmed by a protein degradation assay. The cells that were treated with Ang II showed increased Bcl-x_L protein degradation compared to the control and HGF treated cells (Fig 3B). Co-immunoprecipitation assays demonstrated that while Ang II significantly lowered the Bax and Bcl-x_L protein interaction, which result in mitochondrial outer membrane permeabilization, HGF restored this effect (Fig 3C). These data suggest that HGF prevents the Ang II-induced Bcl-x_L downregulation and inhibits cells from undergoing apoptosis.

Previously, we found that the mechanism involved a reduction of the half-life of Bcl-x_L mRNA from ~7 hours in control cells to ~3 hours. The downregulation of Bcl-x_L mRNA and protein levels, results in a decreased ratio of anti-apoptotic to pro-apoptotic proteins. The decrease in the ratio is sufficient to induce apoptosis (23). Therefore, we investigated the effect of HGF on Bcl-x_L mRNA stability. While Ang II caused a reduction in Bcl-x_L mRNA half-life, the level of Bcl-x_L mRNA in the cells treated with HGF was stabilized even in the presence of Ang II treatment (Fig 3D). These data suggest that HGF can stabilize Bcl-x_L mRNA and prevent the Ang II-induced decrease of Bcl-x_L protein levels.

HGF activated Erk1/2 inhibits Ang II-induced apoptosis. To confirm that HGF utilizes the Erk1/2 pathway to localize nucleolin in the cytoplasm and allow binding and stabilization of protein to bind to and stabilize the Bcl-x_L mRNA and inhibit intrinsic apoptosis, PAEC were treated with Ang II and HGF with either U0126 or LY294002. Ang II treatment alone induced apoptosis while HGF pretreatment

inhibited the cell death (Fig 4A). U0126 and HGF treatment prior to Ang II significantly increased the apoptotic level. LY294002 and HGF treatment prior to Ang II also increased the level of apoptosis, but not to the degree of the U0126 treatment (Fig 4A). When treated with both U0126 and LY294002 treatment prior to HGF and Ang II, the level of apoptosis was comparable to that of Ang II treatment alone (Fig 4A). Similar results were seen when using cleaved caspase 3 as a marker of apoptosis (Fig 4B).

HGF protects the interaction between cytoplasmic nucleolin and the 3'UTR of the Bcl-xL mRNA. Nucleolin protein has shown to bind to 3'UTR of Bcl-x_L mRNA to stabilize and protect mRNA from degradation. We have recently shown that Ang II causes displacement of nucleolin from the Bcl-x_L mRNA (Lee *et al.* 2010. In press). To test if HGF will prevent Ang II-induced displacement, RNA-IP was performed to detect the nucleolin-Bcl-x_L mRNA complex. Ang II decreased the level of Bcl-x_L mRNA bound to nucleolin. HGF pretreatment maintained the Bcl-x_L-nucleolin complex at basal levels (Fig 5A). To confirm our findings, we tested the effects of HGF using a renilla-Bcl-x_L 3'UTR construct. Ang II decreased the levels of renilla-Bcl-x_L, HGF pretreatment inhibited the Ang II-induced decrease (Fig 5B). This data suggest that HGF increases the interaction between cytoplasmic nucleolin and Bcl-x_L mRNA, even in the presence of Ang II.

HGF blocks Ang II-induced translocation of nucleolin to the nucleus. We hypothesize that Ang II-induced nucleolin nuclear translocation caused cytoplasmic

Bcl-x_L mRNA instability and decreased Bcl-x_L mRNA half-life; however, HGF will prevent the relocation of nucleolin proteins, allowing the binding between the nucleolin and the 3' UTR of Bcl-x_L mRNA resulting in the restoration of mRNA stability. To test if HGF can inhibit the Ang II-induced nucleolin translocation, cells were treated with HGF prior to treatment with Ang. Nuclear and cytoplasmic fractions were examined for nucleolin protein using western blot analysis. Treatment with 10 μM Ang II for 24 hours decreased the nucleolin protein in the cytoplasm while it increased in the nucleus (Fig 6). A 30- minute pretreatment with HGF (25 ng/ml) inhibited the Ang II-induced translocation of nucleolin from the cytoplasm to the nucleus (Fig 6). This suggests that HGF inhibits Ang II-induced nucleolin nuclear localization.

HGF stabilization of the binding of nucleolin to the Bcl-x_L 3'UTR requires Erk1/2 pathway.

Prior studies of HGF-induced cell survival suggested the involvement of two major pathways: the Erk1/2 and PI3K/Akt pathway (24,25). To determine which pathway HGF utilizes for stabilization of the nucleolin- Bcl-x_L mRNA complex, cells were treated with either the Mek1/2 inhibitor U0126 or PI3K inhibitor LY294002 prior to the addition HGF ± Ang II. Treatment with Ang II decreased levels of nucleolin in the cytoplasm and increased levels of nucleolin were found in the nucleus (Fig 7A). This was prevented by HGF. Pretreatment with U0126 blocked HGF localization of the nucleolin in the cytoplasm, and nucleolin increased in the nucleus (Fig 7A,B). LY294002 treatment did not effect the HGF inhibition of Ang II-induced

nucleolin translocation (Fig 7A). To determine whether Erk1/2 pathway is required for the interaction between nucleolin and Bcl-x_L mRNA, RNA-IP nucleolin was performed. U0126 pretreatment blocked HGF inhibition of Ang II-induced dissociation of nucleolin and Bcl-xL mRNA. (Fig 7C). Again, LY294002 treatment did not alter the level of nucleolin- Bcl-x_L mRNA interaction induced by HGF (Fig 7C). These findings were confirmed using the renilla-Bcl-x_L 3'UTR. We observed that levels of Bcl-x_L 3'UTR level increased with HGF treatment but U0126 treatment prevented the increase (Fig 7D). These data suggest that HGF-activated Erk1/2 is required for HGF-induced cytoplasmic localization of nucleolin.

HGF inhibits Ang II-induced apoptosis in ex vivo

The *ex vivo* lung slices represent an alternative to *in vivo* method in measuring cellular signaling mechanisms. This technique has the advantage of studying specific organ without the confounding whole body effects (26). Studies have shown that data from the *ex vivo* model can be an strong representative for the *in vivo* models data (26,27). In the *ex vivo* method, an isolated organ sustains the architecture and functionality of the tissue, thus represents a closer model to *in vivo* than the *in vitro* monolayer models from a single cell type. Studies have shown that alveolar cells divide and correctly differentiate in *ex vivo* lung slice cultures (28). *Ex vivo* technique has been used to determine drug responses, apoptosis, and cell survival (29-31). The *ex vivo* lung model also has been demonstrated to act as a reliable model for *in vivo* studies to demonstrate the role of Ang II signaling in lung cell apoptosis from pro-fibrotic stimuli (29,32).

We used the *ex-vivo* lung explant model with Ang II treatment overnight with or without HGF. Subsequently, a LDH-cytotoxicity assay was performed, which evaluates cell death by the quantification of plasma membrane damage. Ang II treatment induced a ~2.4 fold increase in LDH levels compared to the untreated control (Fig 8A). Co-treatement with HGF showed LDH levels comparable to the untreated control (Fig 8A).

With *ex-vivo* lung explants, we found Ang II-activation of pro-apoptotic proteins and HGF inhibition of caspase cascade just like the cell culture model. While the level of cleaved caspase 3 was high in the Ang II treated rat lung explant, the co-treatment with the HGF inhibited the activation of the effector caspase (Fig 8B). Ang II treatment also lowered the level of Bcl-x_L protein level, but with HGF, the level of Bcl-x_L was similar to the control (Fig 8B). Immunohistochemistry on the Ang II treated rat lung tissues revealed high levels of cleaved caspase 3 while HGF treatment lowered the cleaved caspase 3 levels (Fig 8C).

HGF activated Erk1/2 inhibits Ang II-induced apoptosis in ex vivo.

To confirm that HGF activation of Erk1/2 is necessary to inhibit Ang II induced apoptosis, we first utilized chemical Mek inhibitor U0126. Ang II treatment of *ex-vivo* rat lung explants increased levels of cleaved caspase 3 (Fig 9A) but pretreatment with HGF prevented cleavage of caspase 3 (Fig 9A). When the lung slices were pretreated with U0126 prior to HGF and Ang II treatment, levels of active caspase 3 increase (Fig 9A). To verify that U0126 can enter and inhibit MEK in the tissue, a control experiment was performed, showing that phosphorylated Erk1/2 is

present in the tissue and can be inhibited by U0126 (Fig 9A). Lastly, the tissue was infected with either adenovirus-encoded GFP or adenovirus-encoded dominant negative (DN) Mek. Adenovirus-GFP was used to confirm viral incorporation and gene expression in the lung *ex-vivo* explants (Fig 9B). After infection, lung explants were treated with HGF and Ang II. Tissue was homogenized and protein lysates were western blotted to determine the levels of the cleaved caspase 3. Adenovirus-GFP tissues treated with Ang II showed increased active caspase 3 levels; but with HGF treatment, cleaved caspase 3 levels were significantly reduced (Fig 9B). Adenovirus-DN Mek-infected tissue showed increased levels of caspase 3 activation in the presence of Ang II as well as HGF (Fig 9B). Together, the data suggest that HGF utilizes the Erk1/2 pathway to phosphorylate and localize the nucleolin protein in the cytoplasm.

DISCUSSION

The primary finding of this work is that HGF protects lung cells from Ang II-induced apoptosis via activated Erk1/2-mediated localization of nucleolin, which stabilizes Bcl-x_L mRNA, thus inhibiting the intrinsic apoptotic pathway. Our data demonstrate that cytochrome *c* release from the mitochondria, caspase 3 activation and DNA fragmentation are blocked by HGF in both the *in vitro* and *ex-vivo* models. This blockade involves the stabilization of anti-apoptotic Bcl-x_L mRNA via the binding of nucleolin as a result of HGF treatment. And we demonstrated that the binding of nucleolin depends on the activation of Erk1/2. These data confirm that HGF inhibits Ang II-induced intrinsic apoptosis.

The multifunctional nucleolin protein can bind to RNA and DNA (33). In the cytoplasm, nucleolin plays a role in cell survival through Bcl-2 and Bcl-x_L mRNA stabilization (33,34). Studies have shown that the AU-rich region of the 3' UTR of Bcl-x_L and Bcl-2 mRNA is the critical site for stability of the message, as the nucleolin binding to the 3' UTR prevents AU-targeting of the mRNA for degradation (18,33). We have previously shown that while nucleolin does bind to Bcl-2 mRNA in PAEC, Ang II treatment does not decrease the Bcl-2 protein level suggesting Bcl-2 protein does not play a major role in Ang II-induced apoptosis. Upon dephosphorylation of nucleolin, it is translocated from the cytoplasm to the nucleus, where it is no longer available for mRNA stabilization (20). Down-regulation of nucleolin, by siRNA or by agents that cause its degradation, is sufficient to induce growth arrest and apoptosis (35,36). Previously, we found that Ang II caused a decrease in Bcl-x_L half-life by reducing the nucleolin binding to the second AU-rich region of the UTR of Bcl-x_L mRNA (Lee *et al.* 2010. In press).

In the cytoplasm, nucleolin plays a role in cell survival through Bcl-x_L mRNA stabilization; while in the nucleus, it is shown to be related to pro-apoptotic activities (20). Thus, cellular localization of nucleolin is an important factor in cell survival mechanisms. We have shown that Ang II causes nucleolin to translocate to the nucleus while HGF treatment prevented the nuclear localization. The data suggest that HGF-mediated phosphorylation of nucleolin prevents nuclear localization; and the cytoplasmic nucleolin was shown to bind the 3'UTR mRNA of Bcl-x_L. This confirms that HGF facilitates interaction between the Bcl-x_L mRNA and nucleolin even in the presence of Ang II treatment.

The mechanism of HGF-induced cell growth and inhibition of apoptosis involves two major pathways: the Erk1/2 and PI3K/Akt pathways (24,25). HGF activation of Erk1/2 pathway led to an increase of Bcl-x_L protein involving ETS transcription factors in the mesothelimoa cells (37). The PI3K/Akt pathway is associated with cell motility (38-40) and survival (41,42) and has shown to be involved in upregulation of Bcl-x_L protein to protect epidermal keratinocytes from apoptosis *in vivo* (43) HGF has shown the ability to promote cell survival by dual mechanisms involving phosphorylation and deactivation of pro-apoptotic Bad protein and upregulating the anti-apoptotic Bcl-x_L protein (44). Previous studies have shown Akt to exert its anti-apoptotic effects through the Akt phosphorylation of nucleolin (45,46). Akt has also been shown to prevent apoptosis via phosphorysation of the pro-apoptotic protein Bad, which in its unphosphorylated state sequesters and inactivates various anti-apoptotic proteins (47). However, phosphorylated Bad binds with the 14-3-3 protein, which frees anti-apoptotic Bcl-2 proteins to bind and inhibit Bax thus preventing apoptosis (48). However, PI3K inhibition did not cause nuclear localization of nucleolin suggesting that PI3K activity is not necessary to phosphorylate nucleolin.

The cell proliferation and survival effects by HGF (49,50) are initiated by the activation of the Gab1/Grb2/Ras pathway (51), which results in the phosphorylation of Erk1/2 (52,53). Previous studies have demonstrated that HGF-induced Erk1/2 activation prevents cell death (54,55). Studies have also reported that Erk1/2 increases cell survival by regulating the phosphorylation state of anti-apoptotic proteins (56,57) and decreasing levels of caspase 3 activity (58). Erk1/2 inhibition did cause nuclear

localization of nucleolin suggesting that Erk1/2 activity is necessary to phosphorylate nucleolin. Erk1/2 activation was found necessary to induce interaction between nucleolin and Bcl-x_L mRNA while the PI3K/Akt pathway inhibition did not downregulate the interaction between nucleolin and Bcl-x_L mRNA. This data suggests that Erk1/2 activation is necessary in phosphorylating, localizing nucleolin in the cytoplasm, and inducing a nucleolin- Bcl-x_L mRNA interaction. Overall, our data suggest that the HGF-activated Erk1/2 pathway phosphorylates nucleolin and stabilizes the Bcl-x_L mRNA.

Even with the HGF treatment, Erk1/2 inhibition allowed significant increase of Ang II-induced caspase 3 activation and apoptosis. This suggests that HGF utilizes the Erk1/2 pathway to prevent Ang II-mediated apoptosis. However, in the LY294002/HGF/Ang II treatment group, caspase 3 activation was still higher than the untreated group. This suggests that HGF might be also activating PI3K pathway for cell survival. Inhibiting HGF-induced activation of both PI3K/Akt and Erk1/2 allowed Ang II to activate caspase 3 to the level comparable to the Ang II treatment alone. We can conclude that while HGF utilizes Erk1/2 to phosphorylate nucleolin and control the anti-apoptotic Bcl-x_L level in the cell, HGF also utilizes PI3K/Akt pathway for cell survival. Because we demonstrated that PI3K pathway is not involved in nucleolin phosphorylation, PI3K pathway might inhibit MOMP through the phosphorylation of Bad.

Footnotes:

We thank Dr. S. B. Bausch and Mr. W. B. Rittase (USUHS, Bethesda, MD) for rat lung tissue and help in development of the *ex vivo* lung culture technique. We would also like to thank Dr. T. Bowden for providing the renilla-Bcl-x_L construct and Dr. Y. J. Suzuki for providing the dominant negative-Mek adenovirus. This work was supported by NIH HL-073929 and a USUHS research grant to RMD, and by a predoctoral fellowship from the American Heart Association to YHL. Some of the authors are employees of the U.S. Government. This work was prepared as part of their official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government'. Title 17 U.S.C §101 defined a U.S. Government work as a work prepared by a military service member or employees of the U.S. Government as part of that person's official duties. The views in this article are those of the authors and do not necessarily reflect the views, official policy, or position of the Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, or the U.S. Federal Government.

Abbreviations: PAEC, pulmonary artery endothelial cells; FBS, fetal bovine serum; Ang II, Angiotensin II; BSA, bovine serum albumin; HGF, hepatocyte growth factor;

Bibliography:

1. Rubin, J. S., Bottaro, D. P., and Aaronson, S. A. (1993) *Biochim Biophys Acta* **1155**(3), 357-371.
2. Dohi, M., Hasegawa, T., Yamamoto, K., and Marshall, B. C. (2000) *American journal of respiratory and critical care medicine* **162**(6), 2302-2307.
3. Kawaida, K., Matsumoto, K., Shimazu, H., and Nakamura, T. (1994) *Proceedings of the National Academy of Sciences of the United States of America* **91**(10), 4357-4361.
4. Kitta, K., Day, R. M., Ikeda, T., and Suzuki, Y. J. (2001) *Free radical biology & medicine* **31**(7), 902-910.
5. Kosai, K., Matsumoto, K., Funakoshi, H., and Nakamura, T. (1999) *Hepatology* **30**(1), 151-159.
6. Yaekashiwa, M., Nakayama, S., Ohnuma, K., Sakai, T., Abe, T., Satoh, K., Matsumoto, K., Nakamura, T., Takahashi, T., and Nukiwa, T. (1997) *American journal of respiratory and critical care medicine* **156**(6), 1937-1944.
7. Ebina, M., Shimizukawa, M., Narumi, K., Miki, M., Koinuma, D., Watabe, M., Munakata, H., and Nukiwa, T. (2002) *Chest* **121**, 32S-33S
8. Watanabe, M., Ebina, M., Orson, F. M., Nakamura, A., Kubota, K., Koinuma, D., Akiyama, K., Maemondo, M., Okouchi, S., Tahara, M., Matsumoto, K., Nakamura, T., and Nukiwa, T. (2005) *Mol Ther* **12**(1), 58-67
9. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96**(6), 857-868

10. Lee, Y. H., Suzuki, Y. J., Griffin, A. J., and Day, R. M. (2008) *Am J Physiol Lung Cell Mol Physiol* **294**(4), L778-786

11. Wynn, T. A. (2008) *J Pathol* **214**(2), 199-210

12. Konigshoff, M., Wilhelm, A., Jahn, A., Sedding, D., Amarie, O. V., Eul, B., Seeger, W., Fink, L., Gunther, A., Eickelberg, O., and Rose, F. (2007) *Am J Respir Cell Mol Biol* **37**(6), 640-650

13. Filippatos, G. S., Gangopadhyay, N., Lalude, O., Parameswaran, N., Said, S. I., Spielman, W., and Uhal, B. D. (2001) *Am J Physiol Lung Cell Mol Physiol* **281**(4), L749-761

14. Marshall, R. P., Gohlke, P., Chambers, R. C., Howell, D. C., Bottoms, S. E., Unger, T., McAnulty, R. J., and Laurent, G. J. (2004) *American journal of physiology* **286**(1), L156-164

15. Wang, R., Ramos, C., Joshi, I., Zagariya, A., Pardo, A., Selman, M., and Uhal, B. D. (1999) *Am J Physiol* **277**(6 Pt 1), L1158-1164

16. Wang, R., Ibarra-Sunga, O., Verlinski, L., Pick, R., and Uhal, B. D. (2000) *Am J Physiol Lung Cell Mol Physiol* **279**(1), L143-151

17. Wang, R., Zagariya, A., Ibarra-Sunga, O., Gidea, C., Ang, E., Deshmukh, S., Chaudhary, G., Baraboutis, J., Filippatos, G., and Uhal, B. D. (1999) *Am J Physiol* **276**(5 Pt 1), L885-889

18. Zhang, J., Tsaprailis, G., and Bowden, G. T. (2008) *Cancer Res* **68**(4), 1046-1054

19. Srivastava, M., and Pollard, H. B. (1999) *Faseb J* **13**(14), 1911-1922

20. Schwab, M. S., and Dreyer, C. (1997) *European journal of cell biology* **73**(4), 287-297

21. Kitta, K., Day, R. M., Ikeda, T., and Suzuki, Y. J. (2001) *Free Radic Biol Med* **31**(7), 902-910

22. Krown, K. A., Page, M. T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K. L., Glembotski, C. G., Quintana, P. J. E., and Sabbadini, R. A. (1996) *J Clin Invest* **98**, 2854-2865

23. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000) *Science (New York, N.Y* **290**(5493), 989-992

24. Day, R. M., Cioce, V., Breckenridge, D., Castagnino, P., and Bottaro, D. P. (1999) *Oncogene* **18**(22), 3399-3406

25. Fan, S., Ma, Y. X., Wang, J. A., Yuan, R. Q., Meng, Q., Cao, Y., Laterra, J. J., Goldberg, I. D., and Rosen, E. M. (2000) *Oncogene* **19**(18), 2212-2223

26. Henjakovic, M., Martin, C., Hoymann, H. G., Sewald, K., Ressmeyer, A. R., Dassow, C., Pohlmann, G., Krug, N., Uhlig, S., and Braun, A. (2008) *Toxicol Sci* **106**(2), 444-453

27. Defer, N., Wan, J., Souktani, R., Escoubet, B., Perier, M., Caramelle, P., Manin, S., Deveaux, V., Bourin, M. C., Zimmer, A., Lotersztajn, S., Pecker, F., and Pavoine, C. (2009) *Faseb J* **23**(7), 2120-2130

28. Kinnard, W. V., Tuder, R., Papst, P., and Fisher, J. H. (1994) *American journal of respiratory cell and molecular biology* **11**(4), 416-425

29. Li, X., Rayford, H., and Uhal, B. D. (2003) *The American journal of pathology* **163**(6), 2523-2530

30. Lang, D. S., Droemann, D., Schultz, H., Branscheid, D., Martin, C., Ressmeyer, A. R., Zabel, P., Vollmer, E., and Goldmann, T. (2007) *Respiratory research* **8**, 43

31. Hackett, T. L., Holloway, R., Holgate, S. T., and Warner, J. A. (2008) *Respiratory research* **9**, 47

32. Mancini, G. B., and Khalil, N. (2005) *Clinical and investigative medicine* **28**(3), 118-126

33. Sengupta, T. K., Bandyopadhyay, S., Fernandes, D. J., and Spicer, E. K. (2004) *The Journal of biological chemistry* **279**(12), 10855-10863

34. Otake, Y., Soundararajan, S., Sengupta, T. K., Kio, E. A., Smith, J. C., Pineda-Roman, M., Stuart, R. K., Spicer, E. K., and Fernandes, D. J. (2007) *Blood* **109**(7), 3069-3075

35. Ugrinova, I., Monier, K., Ivaldi, C., Thiry, M., Storck, S., Mongelard, F., and Bouvet, P. (2007) *BMC Mol Biol* **8**, 66

36. Kito, S., Shimizu, K., Okamura, H., Yoshida, K., Morimoto, H., Fujita, M., Morimoto, Y., Ohba, T., and Haneji, T. (2003) *Biochemical and biophysical research communications* **300**(4), 950-956

37. Cao, X., Littlejohn, J., Rodarte, C., Zhang, L., Martino, B., Rascoe, P., Hamid, K., Jupiter, D., and Smythe, W. R. (2009) *Am J Pathol* **175**(5), 2207-2216

38. Nakanishi, K., Fujimoto, J., Ueki, T., Kishimoto, K., Hashimoto-Tamaoki, T., Furuyama, J., Itoh, T., Sasaki, Y., and Okamoto, E. (1999) *Clin Exp Metastasis* **17**(6), 507-514

39. Royal, I., Fournier, T. M., and Park, M. (1997) *J Cell Physiol* **173**(2), 196-201

40. Bardelli, A., Basile, M. L., Audero, E., Giordano, S., Wennstrom, S., Menard, S., Comoglio, P. M., and Ponzetto, C. (1999) *Oncogene* **18**(5), 1139-1146

41. Chong, Z. Z., and Maiese, K. (2007) *Histol Histopathol* **22**(11), 1251-1267

42. Tabet, F., Schiffrin, E. L., Callera, G. E., He, Y., Yao, G., Ostman, A., Kappert, K., Tonks, N. K., and Touyz, R. M. (2008) *Circ Res* **103**(2), 149-158

43. Umeda, J., Sano, S., Kogawa, K., Motoyama, N., Yoshikawa, K., Itami, S., Kondoh, G., Watanabe, T., and Takeda, J. (2003) *Faseb J* **17**(6), 610-620

44. Zhang, J., Yang, J., and Liu, Y. (2008) *International journal of clinical and experimental pathology* **1**(3), 242-253

45. Barel, M., Balbo, M., Le Romancer, M., and Frade, R. (2003) *Eur J Immunol* **33**(9), 2557-2566

46. Borgatti, P., Martelli, A. M., Tabellini, G., Bellacosa, A., Capitani, S., and Neri, L. M. (2003) *J Cell Physiol* **196**(1), 79-88

47. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) *Science* **278**(5338), 687-689

48. Hsu, S. Y., Kaipia, A., Zhu, L., and Hsueh, A. J. (1997) *Mol Endocrinol* **11**(12), 1858-1867

49. Matsumoto, K., and Nakamura, T. (2001) *Kidney international* **59**(6), 2023-2038

50. Funakoshi, H., and Nakamura, T. (2003) *Clinica chimica acta; international journal of clinical chemistry* **327**(1-2), 1-23

51. Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994) *Cell* **77**(2), 261-271

52. Sergeant, N., Lyon, M., Rudland, P. S., Fernig, D. G., and Delehedde, M. (2000) *J Biol Chem* **275**(22), 17094-17099

53. Herrera, R. (1998) *J Cell Sci* **111 (Pt 8)**, 1039-1049

54. Nakagami, H., Morishita, R., Yamamoto, K., Taniyama, Y., Aoki, M., Matsumoto, K., Nakamura, T., Kaneda, Y., Horiuchi, M., and Ogihara, T. (2001) *Hypertension* **37**(2 Part 2), 581-586

55. Gao, M., Fan, S., Goldberg, I. D., Laterra, J., Kitsis, R. N., and Rosen, E. M. (2001) *J Biol Chem* **276**(50), 47257-47265

56. Ito, T., Deng, X., Carr, B., and May, W. S. (1997) *J Biol Chem* **272**(18), 11671-11673

57. Horiuchi, M., Hayashida, W., Kambe, T., Yamada, T., and Dzau, V. J. (1997) *J Biol Chem* **272**(30), 19022-19026

58. Xiao, G. H., Jeffers, M., Bellacosa, A., Mitsuuchi, Y., Vande Woude, G. F., and Testa, J. R. (2001) *Proc Natl Acad Sci U S A* **98**(1), 247-252

Figure Legends

Figure 1. HGF inhibits Ang II-induced apoptosis in PAEC. PAEC were grown to 80% confluence and starved in 0.01% FBS media overnight. Cells were treated with HGF (25 ng/ml) for 20 minutes prior to 10 μM Ang II for 24 hours. (A) Neutral comet assay on HGF and Ang II-treated PAEC was done. The percent of apoptotic cells are indicated. Data show means, ± S.D. * indicates statistical

significance from control, $p < 0.05$, n = 4. Experiments were repeated at least 4 times.

Figure 2. HGF inhibits Ang II-induced intrinsic apoptosis. (A,B) PAEC were grown to 80% confluence and starved in 0.01% FBS media overnight. Cells were treated with HGF (25 ng/ml) for 20 minutes prior to 10 μM Ang II for 24 hours. (A) Cells were lysed and mitochondria were removed from the cytosolic components. Mitochondria-free lysates were western blotted for cytochrome c and cleaved caspase 3. Right panels indicate densitometry normalized to β-actin; Data show mean band density normalized to actin ± SD. *Indicates statistical significance from control, $p < 0.05$, n = 3. (B) HGF (25ng/ml) treatment shows inhibition of Ang II (10 μM) DNA fragmentation.

Figure 3. HGF inhibits Bcl-x_L mRNA and protein degradation. PAEC were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. (A) Western blot analysis using the cell lyates were performed for Bcl-x_L protein expression. Panel shows densitometry of Bcl-x_L normalized to β-actin. (B) Cells were pre-treated with HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang II for 16 hours. Following the treatment, 50 μg/mL cycloheximide was added. Protein lysates were used in the western blots to determine the expression of Bcl-x_L. (C) PAEC were treated with HGF followed by Ang II and immunoprecipated for Bax and western blotted for Bcl-x_L (D) Cells were pre-treated with HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang

II for 8 hours. Following the treatment, 5 μg/ml actinomycin D was added and at various time points, total RNA was extracted, and the levels of Bcl-x_L mRNA were monitored by RT-PCR. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 4. HGF-activated Erk1/2 is necessary to inhibit apoptosis. (A,B) PAEC were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pre-treated with combinations of U0126 (10 μM), LY294002 (10 μM), and/or HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang II for 24 hours. A. Neutral comet assay was performed. B. Western blot, using the whole cell lysates, was performed to analyze the protein expression of cleaved caspase 3. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 4. Experiments were repeated at least 4 times.

Figure 5. HGF stabilizes Bcl-x_L mRNA via nucleolin binding. PAEC were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pre-treated with HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang II for 24 hours. (A) Cells were fixed, sonicated, and immunoprecipitated with C23-nucleolin antibody. RNA-protein complex was reverse-crosslinked for 2 hours, and proteinase K and DNase were added. Phenol/choroform RNA extraction was performed for cDNA synthesis. PCR was performed for the second AU-rich region of the 3' UTR of Bcl-x_L. Lower

panel shows densitometry of Bcl-x_L mRNA band. Data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. (B) Cells were transfected with renilla-Bcl-x_L 3'UTR and co-transfected with a luciferase vector RSV-Luc construct to normalize for transfection efficiency. 6 hours after transfection, cells were placed in serum-free medium. Cells were pretreatment ± the HGF (25 ng/ml) before treatment with 10 μM Ang II for 16 hours. Luciferase and renilla assays were performed on cell lysates. Data show means ± S.D. *Indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 6. HGF blocks nucleolin translocation to the nucleus. PAEC were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pre-treated with HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang II for 24 hours. After Ang II ± HGF treatment, cytosolic and nuclear fractions were ran on a western blot and analyzed for the nucleolin protein. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 7. HGF localizes cytoplasmic nucleolin via Erk1/2 pathway. PAEC were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pre-treated with HGF (25 ng/ml) for 20 minutes prior to treatment with 10 μM Ang II for 24 hours. (A) PAEC cells were pre-treated with either LY294002 (10 μM) or U0126 (10 μM) for 20 minutes, followed by HGF (25

ng/ml) prior to treatment with 10 μM Ang II for 24 hours. Cytosol and nuclear extracts were ran on a western and analyzed for the nucleolin protein. (B) Cells were pre-treated with U0126 (10 μM) for 20 minutes, followed by HGF (25ng/ml). Cytosol and nuclear extracts were ran on a western and analyzed for the nucleolin and phospho-Erk1/2 protein. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times. (C) Cells were pretreated with U0126 (10 μM) or LY294002 (10 μM) for 20 minutes, followed by HGF (25 ng/ml) and Ang II (10 μM). RNA-IP followed by PCR amplification was performed for the second AU-rich region of the 3' UTR of Bcl-x_L. (D) Cells were transfected with renilla-Bcl-x_L 3'UTR and co-transfected with a luciferase vector RSV-Luc construct to normalize for transfection efficiency. 6 hours after transfection, cells were placed in serum-free medium. Cells were pretreatment ± U0126 (10 μM) before treatment with HGF (25 ng/ml) for 16 hours. Luciferase and renilla assays were performed on cell lysates. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 4. Experiments were repeated at least 4 times.

Figure 8. HGF inhibits Ang II-induced apoptosis in rat lung *ex-vivo* explants. (A,B) The rat lung tissue was sliced and cultured overnight in a media containing 10 μM Ang II ± HGF (25 ng/ml). (A) The following day, the media was used to quantify the LDH level. (B) The tissue was homogenized and the protein lysates were used for western blots to determine the expression level of caspase 3 and

Bcl-x_L. (C) The rat lung was perfused with the media containing Ang II ± HGF for 48 hours. The lung was fixed with 10% neutral formalin. After the tissue was cut into 10 micron sections, it was stained with DAB for cleaved caspase 3. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 4. Experiments were repeated at least 4 times.

Figure 9. HGF-activated Erk1/2 is necessary to inhibit apoptosis in rat lung *ex-vivo* explants. (A) *Ex-vivo* rat lung tissue explants were treated with 10 μM Ang II ± U0126 (10 μM) and/or HGF overnight. The tissue was homogenized and the protein lysates were used for western blots to determine the expression level of cleaved caspase 3. (B) 10% FBS RPMI media containing the adenovirus GFP was perfused continuously into the rat lung for 72 hours before the tissue was fixed and made into 10 micron sections. The GFP pictures were taken using the Olympus microscope at 20 × magnification. The rat lung tissue extracts were also infected with the adenovirus GFP or DN MEK for 72 hours, followed by overnight 10 μM Ang II ± HGF (25 ng/ml) treatment. Tissues were homogenized and cell lysate was used to run western blots. Protein expression of Erk1/2 and cleaved caspase 3 was performed. Data show means, ± S.D. * indicates statistical significance from control, $p < 0.05$, n = 3. Experiments were repeated at least 3 times.

Figure 1

A

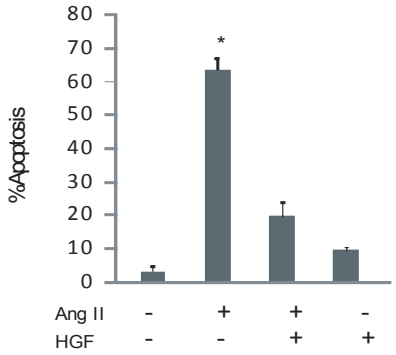
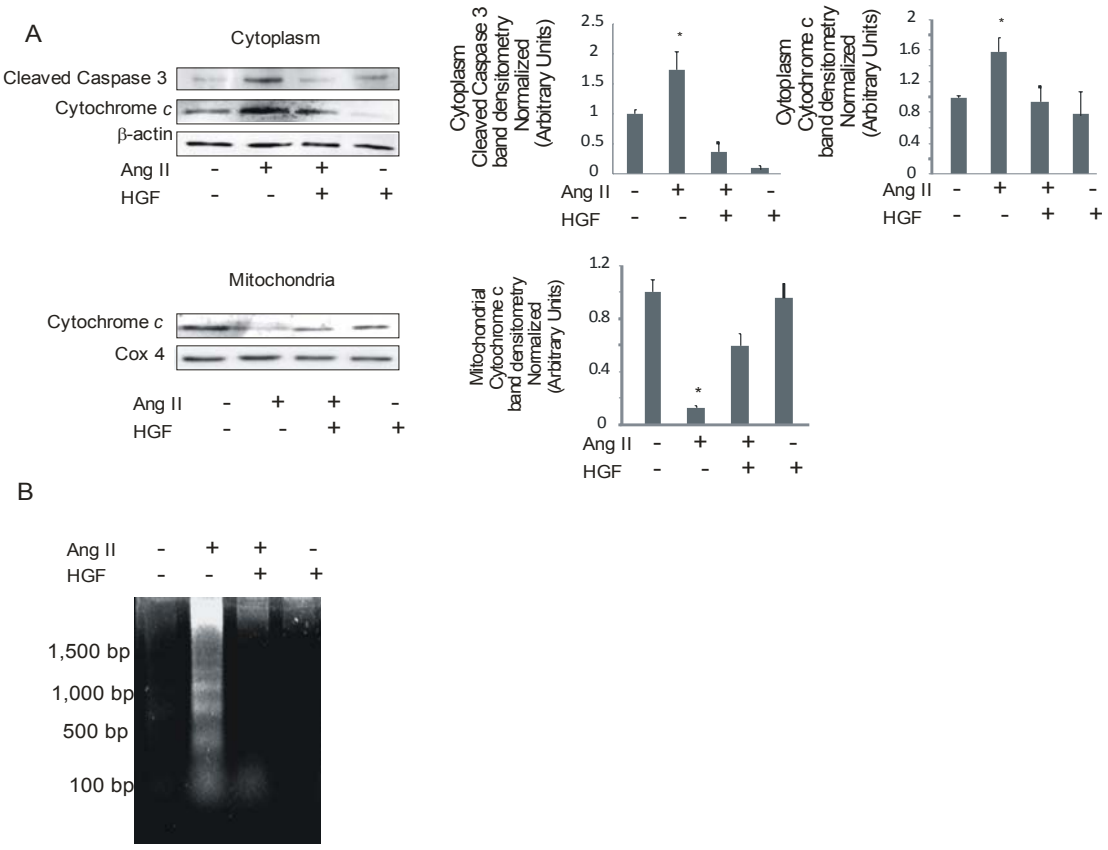


Figure 2


A
Cytoplasm
Cleaved Caspase 3
Cytochrome c
β-actin
Ang II - + + -
HGF - - + +
Mitochondria
Cytochrome c
Cox 4
Ang II - + + -
HGF - - + +
Cytoplasm Cleaved Caspase 3 band densitometry Normalized (Arbitrary Units)
2.5
2
1.5
1
0.5
0
Ang II - + + -
HGF - - + +
Cytoplasm Cytochrome c band densitometry Normalized (Arbitrary Units)
2
1.6
1.2
0.8
0.4
0
Ang II - + + -
HGF - - + +
Mitochondrial Cytochrome c band densitometry Normalized (Arbitrary Units)
1.2
0.8
0.4
0
Ang II - + + -
HGF - - + +

B

Ang II - + + -
HGF - - + +
1,500 bp
1,000 bp
500 bp
100 bp

Figure 3

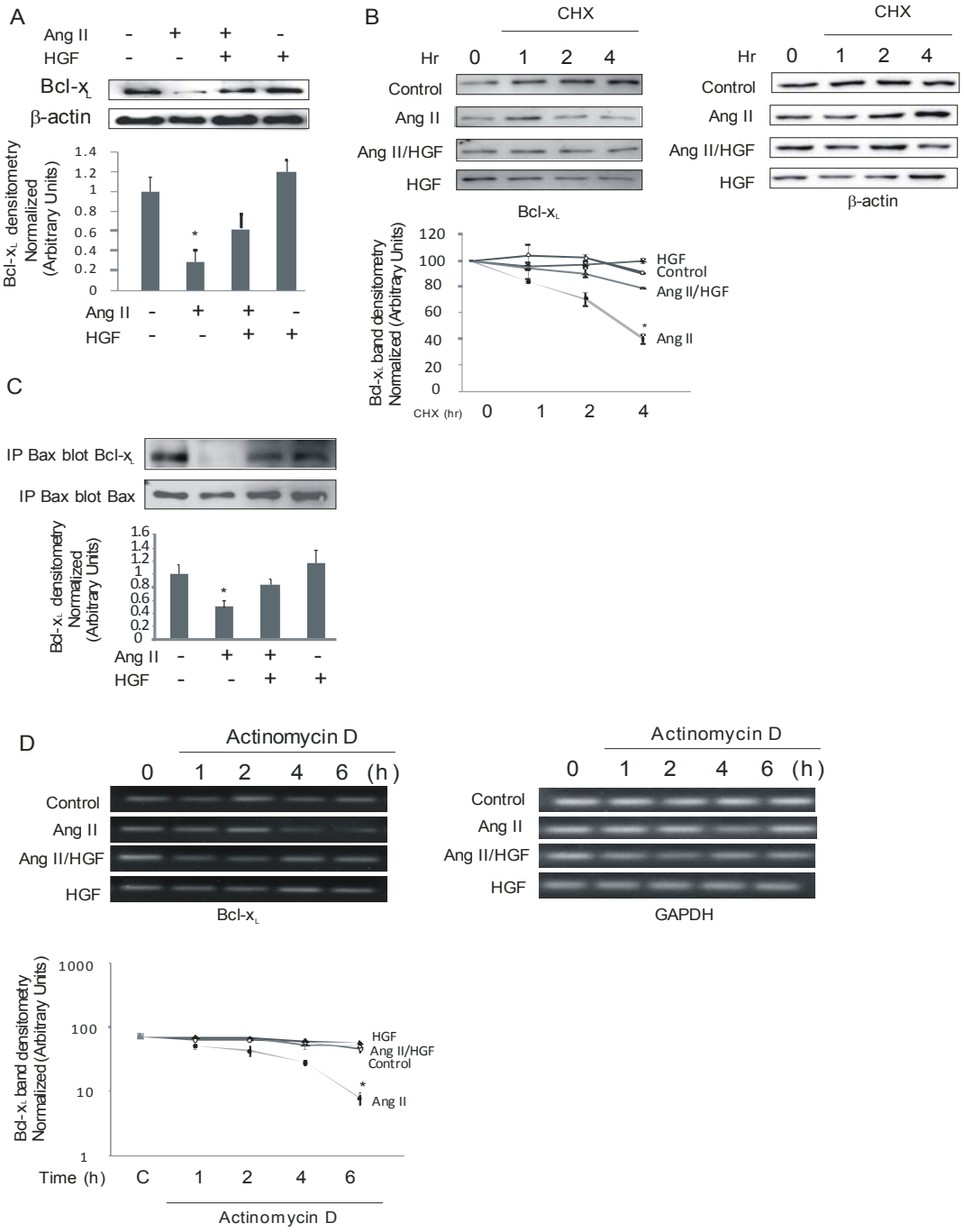

A
Ang II - + + -
HGF - - + +
Bcl-xL
β-actin
Bcl-xL densitometry Normalized (Arbitrary Units)
B
CHX
Hr 0 1 2 4
Control
Ang II
Ang II/HGF
HGF
Bcl-xL
β-actin
Bcl-xL band densitometry Normalized (Arbitrary Units)
HGF
Control
Ang II/HGF
Ang II
CHX (hr) 0 1 2 4
C
IP Bax blot Bcl-xL
IP Bax blot Bax
Bcl-xL densitometry Normalized (Arbitrary Units)
D
Actinomycin D
0 1 2 4 6 (h)
Control
Ang II
Ang II/HGF
HGF
Bcl-xL
GAPDH
Bcl-xL band densitometry Normalized (Arbitrary Units)
Time (h) C 1 2 4 6
Actinomycin D

Figure 4

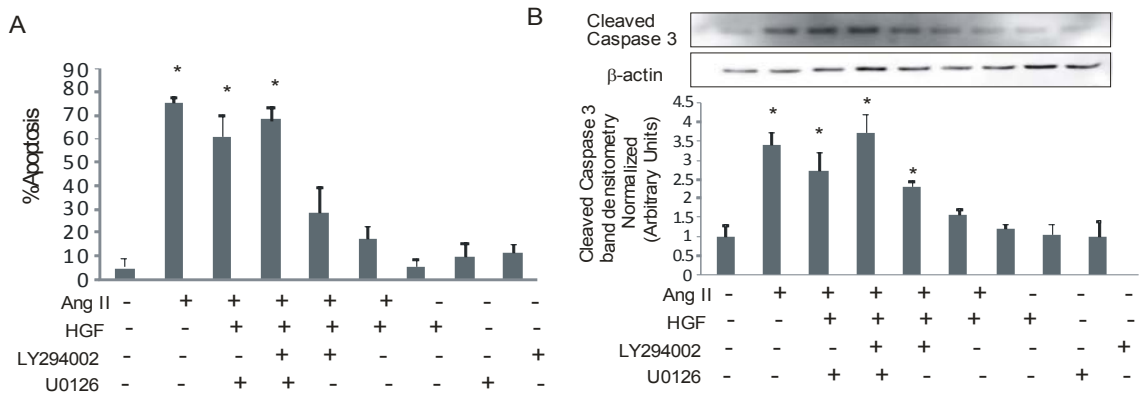

A
%Apoptosis
90
80
70
60
50
40
30
20
10
0
Ang II - + + + + + - - -
HGF - - + + + + + - -
LY294002 - - - + + - - - +
U0126 - - + + - - - + -
B
Cleaved Caspase 3
β-actin
Cleaved Caspase 3 band densitometry Normalized (Arbitrary Units)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Ang II - + + + + + - - -
HGF - - + + + + + - -
LY294002 - - - + + - - - +
U0126 - - + + - - - + -

Figure 5

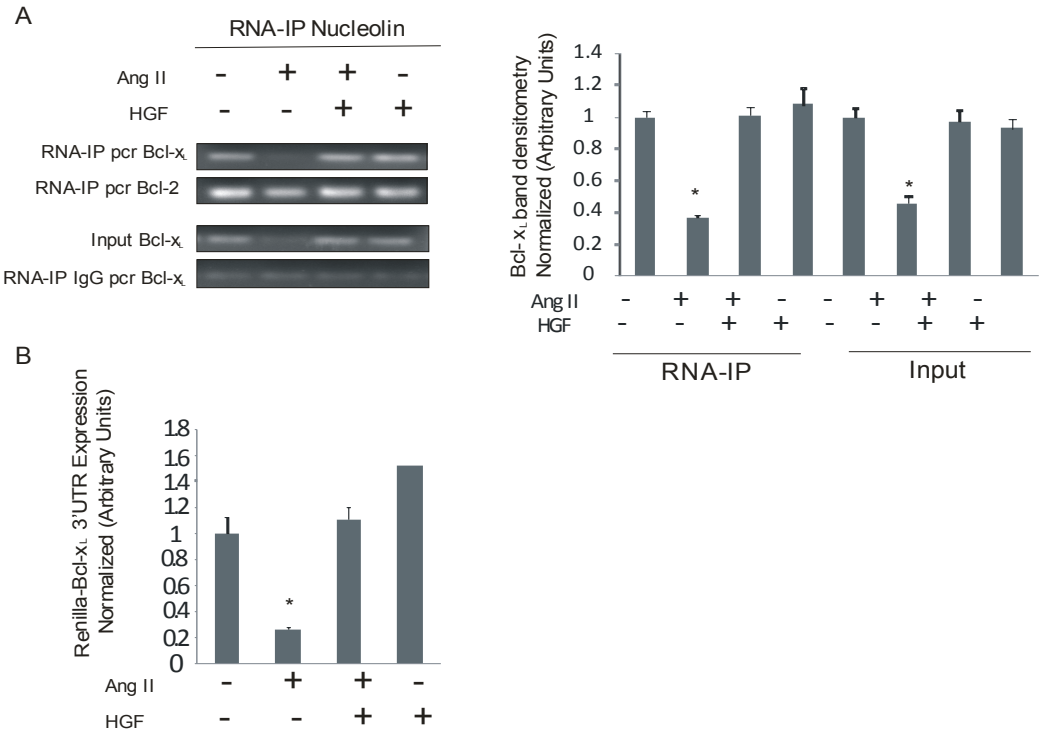

A
RNA-IP Nucleolin
Ang II - + + -
HGF - - + +
RNA-IP pcr Bcl-xL
RNA-IP pcr Bcl-2
Input Bcl-xL
RNA-IP IgG pcr Bcl-xL
Bcl-xL band densitometry Normalized (Arbitrary Units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Ang II - + + - - + + -
HGF - - + + - - + +
RNA-IP
Input
B
Renilla-Bcl-xL 3'UTR Expression Normalized (Arbitrary Units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Ang II - + + -
HGF - - + +

Figure 6

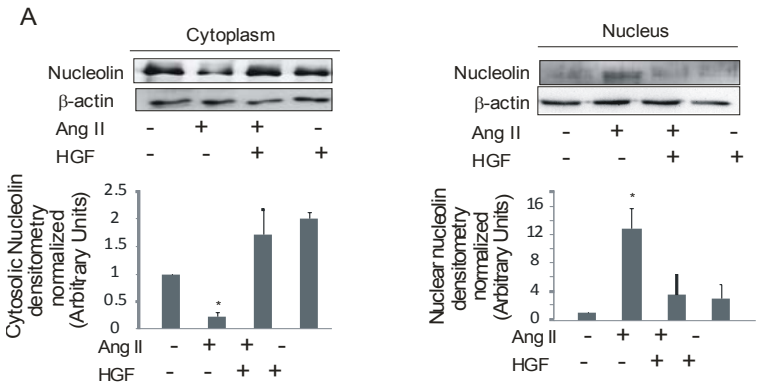

A
Cytoplasm
Nucleolin
β-actin
Ang II - + + -
HGF - - + +
Cytosolic Nucleolin densitometry normalized (Arbitrary Units)
2.5
2
1.5
1
0.5
0
*
Ang II - + + -
HGF - - + +
Nucleus
Nucleolin
β-actin
Ang II - + + -
HGF - - + +
Nuclear nucleolin densitometry normalized (Arbitrary Units)
16
12
8
4
0
*
Ang II - + + -
HGF - - + +

Figure 7

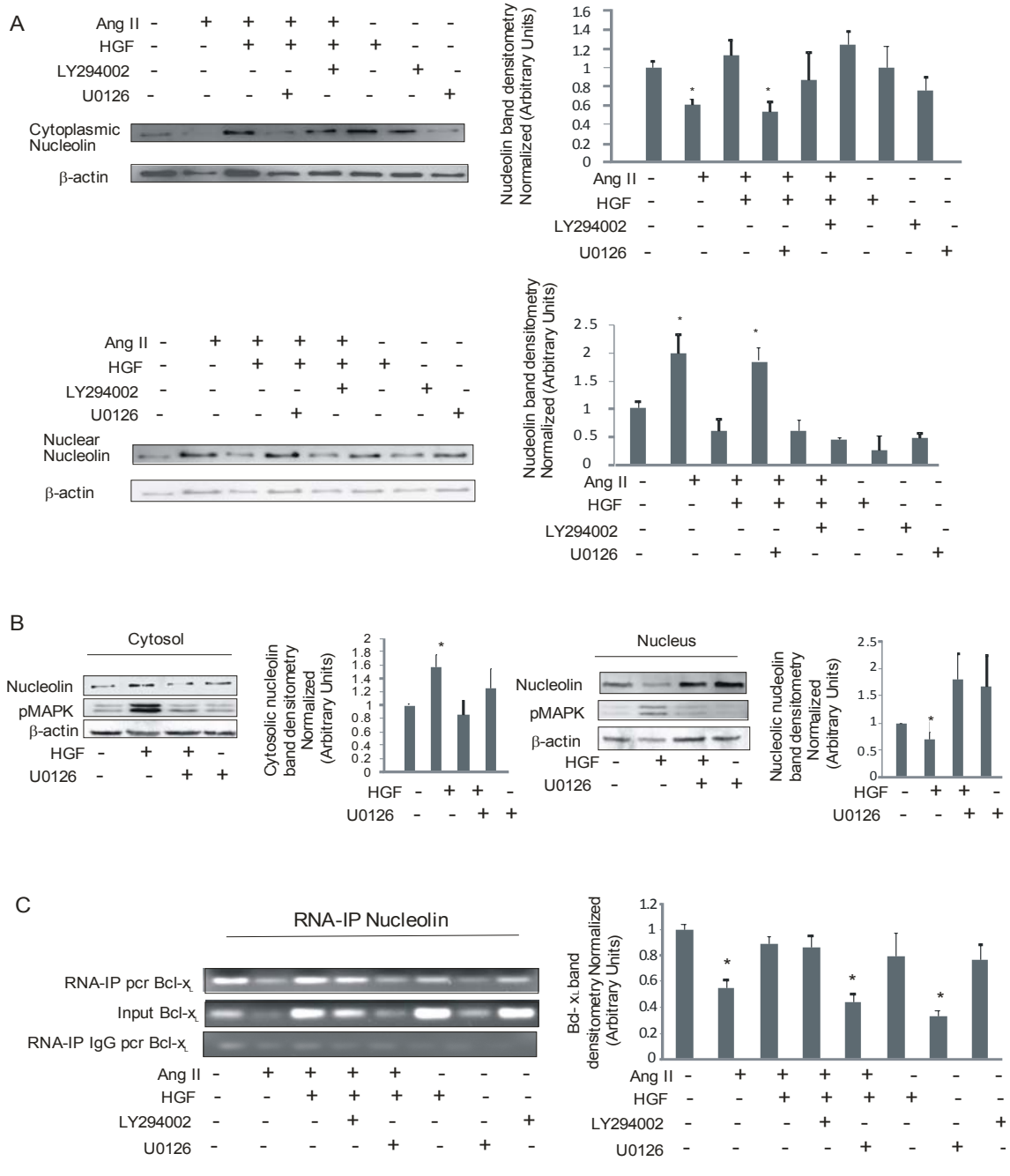

A
Ang II
HGF
LY294002
U0126
Cytoplasmic Nucleolin
β-actin
Nucleolin band densitometry Normalized (Arbitrary Units)
Nuclear Nucleolin
B
Cytosol
Nucleolin
pMAPK
β-actin
Cytosolic nucleolin band densitometry Normalized (Arbitrary Units)
Nucleus
Nucleolic nucleolin band densitometry Normalized (Arbitrary Units)
C
RNA-IP Nucleolin
RNA-IP pcr Bcl-xL
Input Bcl-xL
RNA-IP IgG pcr Bcl-xL
Bcl-xL band densitometry Normalized (Arbitrary Units)

Figure 7 continue

D

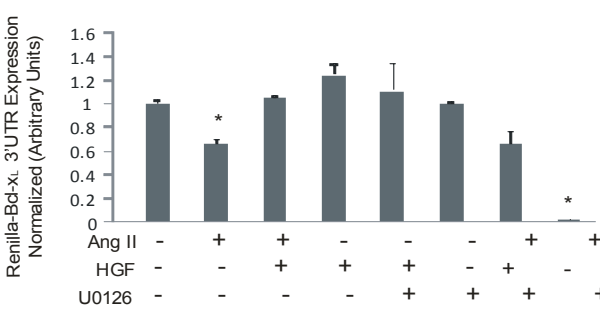


Figure 8

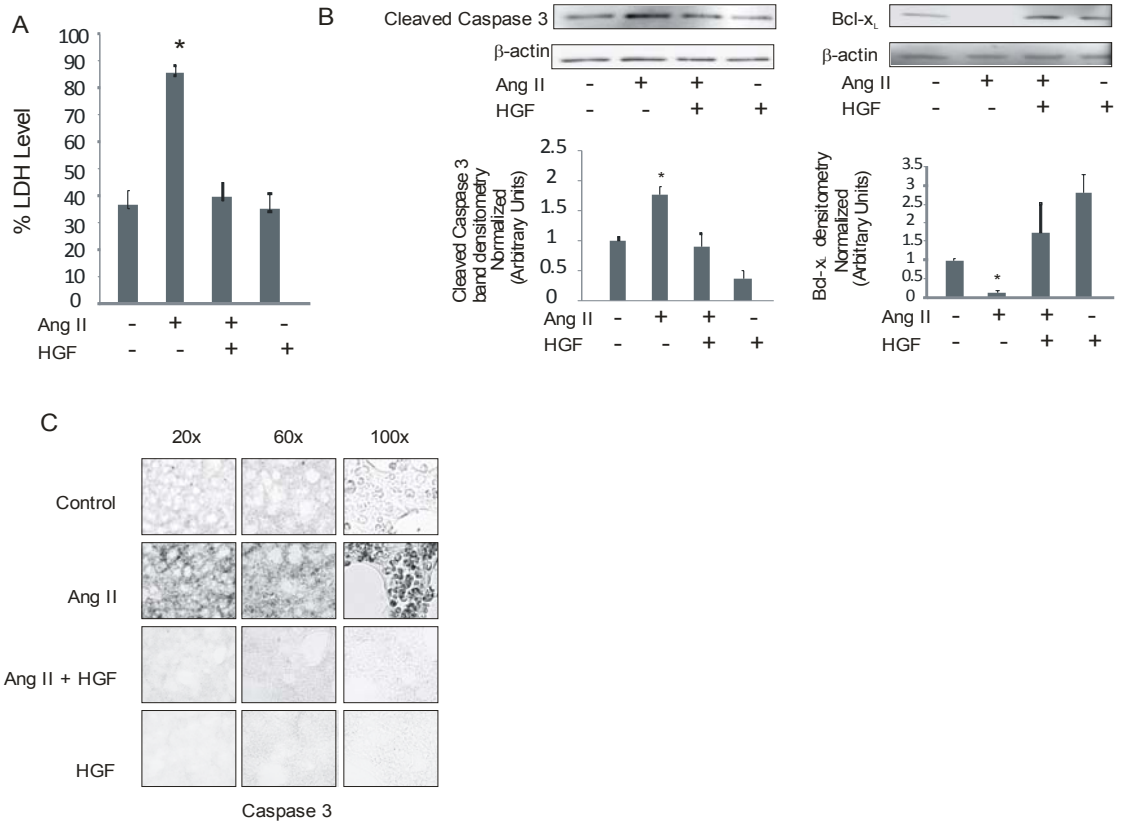
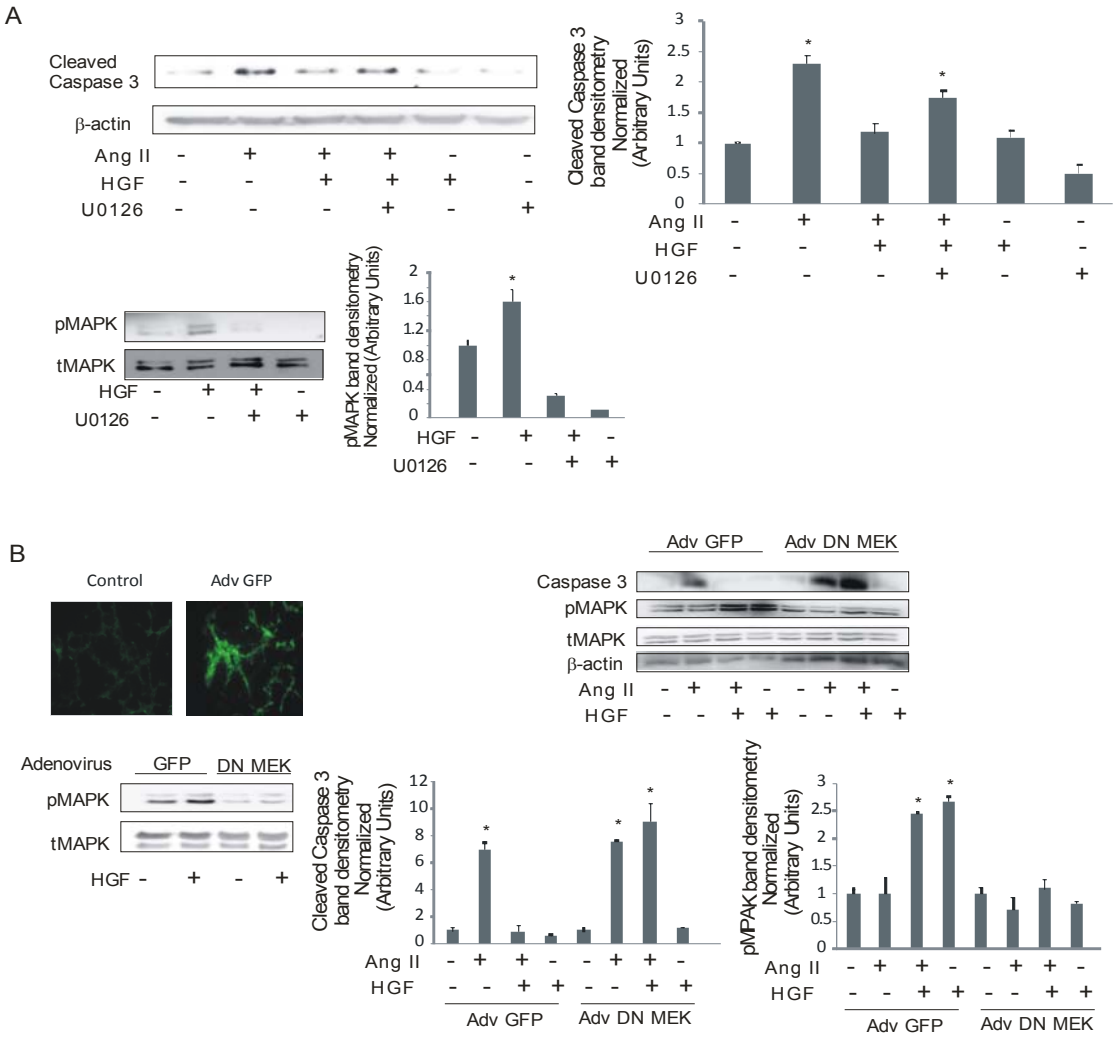

A
% LDH Level
100
90
80
70
60
50
40
30
20
10
0
*
Ang II - + + -
HGF - - + +
B
Cleaved Caspase 3
β-actin
Ang II - + + -
HGF - - + +
Cleaved Caspase 3 band densitometry Normalized (Arbitrary Units)
2.5
2
1.5
1
0.5
0
*
Ang II - + + -
HGF - - + +
Bcl-xL
β-actin
Ang II - + + -
HGF - - + +
Bcl-xL densitometry Normalized (Arbitrary Units)
3.5
3
2.5
2
1.5
1
0.5
0
*
Ang II - + + -
HGF - - + +
C
20x
60x
100x
Control
Ang II
Ang II + HGF
HGF
Caspase 3

Figure 9


A
Cleaved Caspase 3
β-actin
Ang II
HGF
U0126
Cleaved Caspase 3 band densitometry Normalized (Arbitrary Units)
pMAPK
tMAPK
pMAPK band densitometry Normalized (Arbitrary Units)
B
Control
Adv GFP
Adenovirus
GFP
DN MEK
Adv GFP
Adv DN MEK
Caspase 3
pMAPK
tMAPK
β-actin
Cleaved Caspase 3 band densitometry Normalized (Arbitrary Units)
pMAPK band densitometry Normalized (Arbitrary Units)

Chapter 6

Discussion

Pulmonary fibrosis is a case of dysregulated repair and remodification of the lung in response to an injury. In normal tissue, it is critical for the cells to maintain tissue homeostasis by eliminating "abnormal" resident cells that maybe irreparably damaged, infected or defective [478], and this typically occurs through programmed cell death or apoptosis. Additionally, during the resolution phase of the tissue injury, both inflammatory cells and mesenchymal cells that were recruited to repair the injured tissue must be eliminated to restore normal cellular homeostasis and maintain tissue architecture and organ function [103]. Similarly, the apoptosis of myofibroblasts is necessary for the normal resolution of tissue repair responses [103]. However, when the myofibroblasts do not undergo apoptosis, chronic inflammation persists promoting fibroblast proliferation and deposition of extracellular matrix proteins, reflecting dysregulated and exaggerated tissue repair [38]. Overwhelming cellular apoptosis of the epithelial and endothelial cells can also result in unbalanced tissue homeostasis. The loss of alveolar epithelial and endothelial cells, along with the activation and accumulation of myofibroblasts are the key features of the pathophysiology of pulmonary fibrosis [38, 101]. Fibrotic remodeling in the kidney, heart, liver and lung has been shown to involve a coordinated interplay between activated fibroblasts and the loss of normal constituent cells of the tissue [187-190]. Increased levels of angiotensin II (Ang II) have been strongly correlated with both the transdifferentiation of normal fibroblasts to myofibroblasts and the induction of apoptosis in epithelial and endothelial cells [74].

The major finding of this thesis is that Ang II induces apoptosis of primary pulmonary artery endothelial cells (PAEC) by activating the mitochondria-dependent

apoptotic pathway. Apoptosis is induced via the down-regulation of the anti-apoptotic protein Bcl-x_L by affecting its mRNA stability, which occurs as the result of decreased nucleolin binding; however, hepatocyte growth factor (HGF) can inhibit this cellular apoptosis. HGF acts to stabilize the Bcl-x_L mRNA and restore the balance between the pro- and anti-apoptotic proteins.

Angiotensin II-induced apoptosis via the intrinsic apoptotic pathway

The data from this research indicates that Ang II treatment results in the release of cytochrome *c* from the mitochondria, and the activation of initiator caspase 9 and effector caspase 3, ultimately leading to the DNA fragmentation characteristic of cellular apoptosis. This data strongly suggests that Ang II induces apoptosis through an intrinsic apoptotic mechanism.

The permeability of the mitochondrial membrane relies on the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family [128]. In a basal state, the number of pro- and anti-apoptotic molecules is in equalibrium; tipping this balance influence cellular fate. If a stress, such as DNA damage, occurs, the induction of pro-apoptotic molecules provides the signal to engage mitochondrial outer membrane permeabilization (MOMP) [479-481]. On the contrary, addition of growth factors would promote cellular survival by increasing the amount of anti-apoptotic proteins [481-484]. Ang II treatment caused a decrease in the Bcl-x_L mRNA and protein level leading to an increase in the ratio of pro- to anti-apoptotic proteins, resulting in MOMP, and subsequently, apoptosis.

Bax and Bak are essential effector molecules for MOMP; absence of Bax and Bak renders cells resistant to pro-apoptotic stimulations [485, 486]. To hinder the MOMP effects of Bax and Bak, anti-apoptotic proteins can bind and inhibit the function of Bax and Bak to ensure the mitochondrial integrity [480, 487]. Sedlak *et al.* [480] established that Bcl-x_L can heterodimerize with Bax and inhibit apoptosis; the failure to heterodimerize between Bax and Bcl-2 proteins led to cellular apoptosis [487]. The ratio of Bcl-2 proteins to Bax determines dimerization and the vulnerability of a cell to an apoptotic stimulus [481, 488, 489]. A decrease in the ratio of Bcl-2 proteins to Bax leads to apoptosis [480]. Consistent with this, the experimental results from this thesis confirmed that Ang II treatment reduced the Bcl-x_L:Bax interaction, resulting in Bax translocation to the mitochondria, causing the release of cytochrome *c* and inducing apoptosis. Bax channel blockers and Bax inhibiting peptides suppressed Ang II-induced apoptosis. Overall, I concluded that Ang II-mediated reduction of anti-apoptotic Bcl-x_L protein level affected the balance between the Bcl-x_L to Bax, causing Bax to translocate to the mitochondria and inducing MOMP.

Angiotensin II induces apoptosis via AT2

Prior studies have found differing roles of AT1 and AT2 receptors in cell proliferation and apoptosis [196, 226]. The contradictory effect of Ang II-induced cellular proliferation versus apoptosis has been hypothesized to be determined by the specific cell type as well as differences in downstream signaling activated by each

receptor [176, 223, 227, 230-232]. Negative cross-talk between the AT1 and AT2 receptor has also been found [266, 273, 490-493]; for instance in vascular smooth muscle cells, Ang II treatment can activate signals through both AT1 and AT2, where it will lead to an activation of Pyk and ERK/ MAPK for cell survival via the AT1 receptor. This signaling by AT1 is inhibited by SHP-1 phosphatase activation via the AT2 receptor [273, 490]. In PAEC, I have demonstrated that Ang II-induced apoptosis required AT2 but not AT1 activation. AT1 receptor antagonists did not block apoptosis while the AT2 receptor antagonist inhibited Ang II-induced apoptosis. Also, the addition of a specific AT2 agonist resulted in endothelial cell apoptosis. This suggests that Ang II induces apoptosis via the AT2 receptor.

The basis for the preference of Ang II for selection of the AT2 receptor, rather than AT1, to undergo apoptosis during fibrosis is still unclear. Konigshoff *et al.* [199] reported increased expression of both AT1 and AT2 receptors in human lung tissues from patients with IPF and mouse lung tissues derived from the bleomycin-induced model of lung fibrosis. Marshall *et al.* [77, 494] confirmed the finding that AT1 is elevated, but primarily in healthy fibroblasts; Ang II treatment induced migration and proliferation via AT1 receptor in these fibroblast cells. Other studies show that during the development of fibrosis, the Ang II receptor expression undergoes a shift in favor of higher AT2 levels in various cells including the endothelial cells [199, 203, 287]. The higher level of AT2 receptor will tip the Ang II-mediated cellular response to AT2 mediated signaling pathway.

It is also important to note that the binding of Ang II to the AT2 is distinct from that of the AT1 receptor [287]. While the AT1 receptor has a constrained

conformation and can be activated only when bound to tyrosine-4 and phenylalanine-8 residues of Ang II, the AT2 receptor has a 'relaxed' conformation where no single interaction is critical for binding [287]. A previous study by Miura *et al.* also demonstrated that Ang II has a higher affinity for binding to the AT2 receptor [495]. From these studies, we can conclude that the Ang II in the IPF patients will likely bind to AT2 more readily than to the AT1 receptors, resulting in cellular apoptosis of endothelial cells and continuing fibrosis.

Ang II and SHP-2 regulation

There are two main signaling pathways downstream of the AT2 receptor, protein kinase C (PKC) and tyrosine phosphatase pathways [227, 264-266]. I demonstrated that Ang II utilizes the tyrosine phosphatase pathway to induce cellular apoptosis. The activation of SHP phosphatase is one of the major downstream effects of Ang II [266, 273, 490-493]. Feng *et al.* [235] demonstrated in epithelial cells, an inactive G_{as} subunit must be present at the AT2 receptor to activate SHP-1. However, as shown in this thesis as well as by various other studies [297-301], only SHP-2 can be found in the endothelial cells; where as in the epithelial cells, both SHP-1 and -2 are present. This thesis demonstrates that in endothelial cells, Ang II requires the presence of the inactive $G_{\alpha s}$ protein for AT2 to activate the SHP-2 phosphatase. Studies have shown that SHP-2 must be tyrosine phosphorylated to become active [496, 497]. I have shown that SHP-2 is phosphorylated upon Ang II treatment within 1 minute, which is confirmed by phosphatase activity assays. My data demonstrate

that SHP-2 is required for Ang II-induced apoptosis and SHP-2 activation is necessary for Ang II-mediated decrease of Bcl-x_L protein levels. The data strongly suggests that Ang II utilizes SHP-2 to induce cellular apoptosis.

Studies have shown that the adaptor protein growth receptor-bound protein 2 (Grb2) recruits and activates various proteins downstream of the AT2 receptor [280, 284, 498]. To determine if Grb2 plays a role in Ang II-mediated signaling in PAEC, co-immunoprecipitation of SHP-2 and Grb2 was done. I found that when the PAEC were treated with Ang II, Grb2 immediately interacts with SHP-2 (unpublished finding), suggesting that Grb2 adaptor protein is present during SHP-2 activation.

Activation of AMPK and Apoptosis

Next, I wanted to determine the kinase that was responsible for SHP-2 activation. A study by Pan *et al.* [499] demonstrated that Grb2 recruits, interacts, and mediates the phosphorylation of AMP-activated protein kinase (AMPK). AMPK plays an important role in the regulation of energy homeostasis [293]. The kinase is activated by an elevated AMP/ATP ratio due to various cellular and environmental stresses, such as hypoglycemia, oxidative stress, anoxia, hypoxia, heat shock, ischemia, and DNA damage [293]. While previous studies have demonstrated the requirement of energy generation in the form of ATP to induce apoptosis [500-502], the signaling mechanism for apoptosis-dependent ATP generation was unfound. My unpublished co-immunoprecipitation data demonstrate that SHP-2 interacts with AMPK upon Ang II treatment. Furthermore, Ang II-induced apoptosis requires

AMPK activation that occurs downstream of the AT2 receptor and not the AT1 receptor in PAEC (unpublished finding). Inhibition of AMPK activation by the AMPK inhibitor compound C prior to Ang II treatment resulted in decreased SHP-2 phosphorylation and lack of SHP-2 phosphatase activity (unpublished finding). These results strongly suggest that AMPK is the kinase responsible for phosphorylation and activation of SHP-2.

While I found strong evidence that AMPK phosphorylates SHP-2 to induce cellular apoptosis, the activation of the AMPK protein itself brought up the question: why would the activation of an energy generating kinase be necessary in cells that are undergoing apoptosis? Before investigating the question, I confirmed the necessity of energy-dependent apoptosis by treating the cells with compound C or the ATP synthase inhibitor oligomycin prior to the Ang II treatment. Inhibition of AMPK and ATP synthase both suppressed the AT2-induced apoptosis (unpublished finding). This suggests that Ang II-induced apoptosis requires the activation of AMPK for energy production during apoptosis.

Studies have shown that cellular apoptosis requires energy [500-502]. Li *et al.* first demonstrated that ATP is necessary for and can accelerate caspase-3 activation [503]. Also, in the presence of ATP/dATP, Apaf-1 becomes active to recruit the initiator caspase 9 [503]. Liu *et al.* [504] found that dATP can initiate the activation of CPP32 and DNA fragmentation. CPP32 is an apoptotic protein marker that exists in the cytosolic fraction as an inactive precursor that becomes activated in cells undergoing apoptosis [505, 506]. The elevated presence of dATP in Hela cells caused

cleavage and activation of poly (ADP-ribose) polymerase, PARP, and subsequent DNA fragmentation [504].

AMPK activation has also shown the ability to affect the expression of proteins through mRNA stability [507]. Through an unknown mechanism, the activation of AMPK reduces the cytoplasmic level of the RNA-binding protein HuR [507]. HuR has been shown to stabilize specific mRNAs in the cytoplasm by binding to their 3' untranslated regions [507]. The mRNA targets of HuR include proteins that regulate the cell cycle, such as cyclin A and cyclin B1 [507]. By destabilizing and decreasing the mRNA and proteins required for cell cycle progression, the cells will stop their growth and development. Another biosynthetic pathway that is down-regulated by the activation of AMPK is protein translation. Protein translation accounts for ~20% of the energy used in the growing cells and is sensitive to decreases in ATP synthesis [509]. Two possible ways in which AMPK can influence the inhibition of translation have been found. First, the AMPK-mediated phosphorylation and the activation of elongation factor 2 kinase will prevent the elongation step in translation [510, 511]. Second, the activation of AMPK can inhibit the target of rapamycin (TOR) pathway [512-514], which is associated with protein synthesis and cell growth [515-519]. The inhibition of the mTOR pathway by AMPK activation occurs through phosphorylation of TSC2 [520]. The TSC1-TSC2 complex is known to negatively regulate cell growth by inhibiting mTOR [521]. When the phosphorylated TSC2 forms a complex with TSC1 and inhibits mTOR, cell growth and protein synthesis are inhibited and under certain conditions, the cells may undergo apoptosis [521]. Prevention of cell growth along with the inhibition of protein translation results in cellular apoptosis [508].

Ang II-mediated destabilization of Bcl-x_L mRNA

As Ang II treatment resulted in decreased Bcl-x_L protein expression, I wanted to determine if the Bcl-x_L mRNA level was also affected; therefore, the total RNA from Ang II- treated cells was used for qPCR with the Bcl-x_L primers. The result revealed that Ang II does decrease Bcl-x_L mRNA. There are three possible mechanisms that can decrease the Bcl-x_L mRNA: alternative splicing of Bcl-x_L to Bcl-x_S, downregulation of the Bcl-x_L promoter, and Bcl-x_L mRNA decay.

Alternative splicing results in two distinct Bcl-x mRNAs: anti-apoptotic Bcl-x_L and pro-apoptotic Bcl-x_S [522, 523]. Anti-apoptotic Bcl-x_L contains two highly conserved BH1 and BH2 domains that are essential for the anti-apoptotic functions of the Bcl-2 protein family [487]. In pro-apoptotic Bcl-x_S, the conserved BH1 and BH2 domains are absent [487]. This absence is due to the alternative splicing of the common pre-mRNA. While Bcl-x_L displays two complete coding exons, Bcl-x_S lacks the 3' terminal section of the first coding exon [522]. This is due to exploitation of a facultative splice donor site in the first coding exon [487]. To determine whether alternative splicing occured in my system, cells were treated with Ang II and total RNA was isolated. Using the primers for Bcl-x_L and Bcl-x_S, I demonstrated that Ang II does not produce Bcl-x_S mRNA(unpublished data), suggesting that the Ang II-mediated Bcl-x_L mRNA decrease is not due to alternative splicing.

The second possible mechanism is the down-regulation of the Bcl-x_L promoter. The Bcl-x_L promoter is a directed by a complex mechanism which uses alternative

promoters to regulate Bcl-x_L and Bcl-x_S [138]. Grillot *et al.* [524] originally reported that the Bcl-x gene is transcribed from two TATA-less promoters found between -149 and -142 and between -655 and -727. Furthermore, Pecci *et al.* [138] discovered three additional promoters further upstream located at -1886, -2721, and at -3412 relative to the start site and MacCarthy-Morrogh *et al.* [525] demonstrated that these promoters contain a TATA consensus sequence. In prior studies, Bcl-x_L expression was influenced by various transcription factors including E-twenty six (ETS), nuclear factor –κB (NF-κB), and signal transducers and activators of transcription (STATs) [526-529]. To determine whether the Bcl-x_L promoter affects the level of the mRNA, I transfected PAEC with Bcl-x_L-luciferase promoter. However, Ang II treatment did not affect the Bcl-x_L promoter (unpublished findings), suggesting that Ang II-mediated Bcl-x_L mRNA decrease is not through the promoter.

Lastly, an mRNA degradation assay was performed. I demonstrated that Ang II treatment resulted in a decrease in the half-life of Bcl-x_L mRNA from ~7 hours (control) to ~3 hours (Ang II). This reduction of Bcl-x_L mRNA, and thus Bcl-x_L protein levels leads to a decreased ratio of anti-apoptotic proteins to pro-apoptotic proteins, resulting in cellular apoptosis.

Nucleolin binding and stabilization of Bcl-x_L mRNA

Many studies have reported multiple genes that are regulated at the post-transcriptional level through the 3'-untranslated region (3'UTR), such as interleukin-3, β-interferon, junB, c-myc, c-fos, c-jun, granulocyte-macrophage colony-stimulating

factor (GMCSF) and cycoloxygenase-2 (Cox2) [370, 530-538]. One mechanism of mRNA stabilization occurs through adenylate/uridylate-rich elements (AREs) within the 3'UTR that consists of the pentamer AUUUA [381]. Notably, a *cis*-element ARE has demonstrated to be critical for the stability of mRNAs [381]. I have identified three AREs in the Bcl-x_L 3'UTR for RNA-protein binding sites (Figure 10).

A number of RNA-binding protein family members that have been shown to bind to AREs include HuR, nucleolin, ARE/poly-(u) binding/degradation factor 1, far upstream sequence element binding protein, heterogeneous nuclear ribonucleoprotein C, tristetraprolin, TIA-related protein, and T cell restricted intracellular antigen [390-392, 394, 539-541]. Each of these RNA-binding proteins physically interacts and stabilizes the mRNA [217, 390-392, 394, 539]. Specifically, nucleolin has been found to stabilize several messages including Gadd45, Bcl-2, IL-2 and Bcl-x_L [141, 391, 394, 542].

Nucleolin is a multifunctional RNA- and DNA-binding protein that is ubiquitously expressed [543]. Studies have shown that phosphorylated nucleolin plays a role in cell survival by aiding in the stabilization of Bcl-2 and Bcl-x_L mRNA in the cytoplasm [391, 544]. When cytoplasmic nucleolin binds to the 3'UTR of Bcl-x_L, it prevents AU-targeting of the mRNA for degradation [141, 391]. However, when nucleolin is dephosphorylated, the protein is translocated from the cytoplasm to the nucleus [329]. In the nucleus, nucleolin has various activities including nucleogenesis [354], transport of ribosomal components to the cytoplasm [345], rRNA processing

>gi|125991765|ref|NM_001077486.2| Bos taurus BCL2-like 1 (BCL2L1), nuclear gene encoding mitochondrial protein, mRNA

CCGGAAGTGACTGAGCTTGCAAGTTCCCCCGGTCTCTTCAGGGAAACTGAGGCCGGCTCGTTCGGAAGAG
AGAGCGCGAGCCGTCAGCCAGGTAGGCCGGCCCCGGTCCGCAGCGCGGAACTTGGCCGCGAAGAGCACTC
GCGCCCGGAAACGACCTGGCCGATGAAGGGGGTATGTGGCCCCCCACGGCTCGCGGGGCTCGCAGAACCT
TGTCTTGGCTTTGGATCTTCGAAGAGAACCACTAAGCAGAGACCAGACCCAGTGAGTGAGCAGGTGTTTT
GGACAATGGACTGGCGGAGCCCATCCTTATTATAAAAATGTCTCAGAGTAACCGGGAGCTGGTGGTTGAC
TTTCTCTCTTACAAGCTTTCCCAGAAAGGATACAGCTGGAGTCAGTTTAGTGATGTGGAAGAGAACAGAA
CTGAGGCCCCAGAAGGGACAGAATCAGATATGGAAACCCCCAGTGCCATCAATGGCAACGCATCCTGGCA
CCTGGCGGATAGCCCTGCTGTGAATGGAGCCACTGGCCACAGCAGAAGCTCGGATGCCCGGGAAGTGATC
CCCATGGCAGCGGTGAAGCAAGCCCTGAGGGAGGCAGGCGATGAGTTTGAACTGAGGTACCGACGGGCAT
TCAGCGACCTGACGTCCCAGCTCCACATCACCCCAGGGACAGCATATCAGAGCTTTGAACAGGTAGTGAA
TGAACTCTTCCGGGACGGGGTGAACTGGGGTCGCATTGTGGCCTTTTTCTCCTTCGGTGGGGCACTGTGC
GTGGAAAGCGTAGACAAGGAGATGCAGGTATTGGTGAGTCGGATCGCAACTTGGATGGCCACTTACCTGA
ATGACCACCTAGAGCCTTGGATCCAGGAGAACGGCGGCTGGGAGCCTTTTGTGGAACTCTACGGGAACAA
TGCAGCAGCCGAGAGCCGGAAGGGCCAGGAGCGCTTCAACCGCTGGTTCCTGACGGGCATGACTGTGGCT
GGTGTGGTTCTGCTGGGCTCGCTCTTCAGTCGGAAATGACCAGACACTGACCACCAACTCACCCACACAC
CCCCGTCCTGCCTCCACCACATCTCTCCGTCCAGCCACCATTG**CACCAGGAGAACCACTCCAT** GGAGCC
CACAGCCACCCACACATCACAGGGTTGGGCCTAGACCTGATCCCCCGCAATTAGTTTTCTAGAATTTA CA
TGTTTCTGTGAGAG**CC**ACCTTCCTCAATTGTCGTGGCATCAAAACCCACAAAATTCCCTAGAACCTGCCC
CGGTGGAAGCTAACAGGTGTCGGGGGTTGTGACTGGGGGCCGGAGTGCCCCACCCGATTGGTGGGTCCCC
TTCCGCATTTAGGGTCCCTGAGCATGCTTTCTTGCCAGGGAGCTGGAAG**GTTTTCTGACCCTTTTCCCC** A
GAAAGAGAGACAATAGATTGCCTTCATTTTGATGTCTGTGGCCTCAGAATTGATCTATTCCTGTCTCCTC
CCTCCTCCCGGCCCTGGGCCCCCCGTCCATTCATCCCCACCCTCCAAGAGCCACTTAGGACCCACTTCTG
ACTAATTAGGGATTCAGGATGCTTGGGATAAAGAAATAAGGACCAGGACCCCCTCCCCCTCTCTGACCTG
GCCAAAGTCCCTCCCCCCAATCCCAGTGTCCTCTGGAGGTCTCTGGCTAGAGGCCGGCCTCACCCGAGAT
GGAGGGACTGTAGCTGTAGGAAGCACCCCATGCCAAAGCTGGGGTGGCCTTGCAGTTTAGCACCACCCCA
GTTCTTCCCCTTCCCTGGCTCCATGACCATGACTGAGGGACCAACTGGGCTTGAGATAGGTGCCGCAGAG
CCGTTCATGGCCTCAGCTGCCTCACTTCCTGCAAGAAGATGAGCCTGTGGCTTCTTTGCCTTGGGCTGCT
GCCCATGGGGTTCAGGGGCCATGGTCCAGAGGTGAGGGGGAGCTACAGGGAGCAGCTGTGGGAGCCTAG
GGTCTTCCTACCTCAGGCAGGAAGGGCAGGAAGGAGAGCCTTGTGCGTGGGGTGGAGCTGGGATTGGCCG
GAGGGGCCAGCCCTGCCTGGCCTGAACAATTGGTACCCCACCCCCATTCAGCCTGAGCAGCCGGGCTGCC
AAGGTCAGGATGGCCTGGCCAGGAGCTCTTCTGACCTCCCTCTCTCCTCTGCTCCACCCACGGCCTGT
CTCATCCCTGGGGGTCCAGGCCTAGTCCTGGCCACC**CGGGCTCTCTGCTGTACATATT** TGAAACTAGTT
TTTATTCCTTGTGAAGATGATATACTATTTTTGTTAAGCGTGTCTGAATTTA TGTGTGAGGAGCTGCTGG
CTTGCAGTGCGTGTGCACGTGGAGAGCTGGTGCCCGA**AGATCGAACAGCCTGATGCT** CCCTGCACCCCCA
CCCCACCCCACCCCCCGCCCTGGTCTGGGGAAGCCAGCCGGGGGTCCTGGCTCCTGAGAGGCACCTGTCC
CTCCCCTAACCCCCAGCCCACACTTTTTCCAGCTCTTTGAAATAGTCTGTGTGAAGTAAAGGTGCAGTT
CAGTAATAAAATGTGTTCTTCGTGAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 10, Bcl-xL 3'UTR sequence. Nucleotides in red are the ARE biding sites. The bolded nucleotides indicate the primer sequences for the first and the third ARE while the underlined bases represent the primer sequences for the second ARE site.

[341], and chromatin remodeling [545]. The phosphorylation of nucleolin in the nucleus can result in the shuttling of the protein out of the nucleus to the cytoplasm and also alter its sensitivity to proteolysis [356, 543, 546]. When the level of nucleolin is reduced by siRNA or by agents that cause its degradation, cells undergo growth arrest and apoptosis [547, 548].

Through RNA-immunoprecipitation, I revealed that nucleolin binds to the second ARE of the 3'UTR of Bcl-x_L mRNA. This thesis also demonstrates that Ang II-mediated down-regulation of Bcl-x_L is associated with decreased binding of nucleolin to the 3'UTR of Bcl-x_L mRNA. With Ang II treatment, the interaction between nucleolin and Bcl-x_L mRNA decreases, which results in the destabilization and degradation of mRNA. The disruption of the interaction between nucleolin and the 3'UTR region of the Bcl-x_L mRNA is thus the proposed mechanism by which the degradation of the Bcl-x_L mRNA leads to the reduction of Bcl-x_L protein and imbalance of pro- to anti-apoptotic proteins in the cell.

The question of how nucleolin would detach from the 3'UTR of the Bcl-x_L remained unclear. Because I had already demonstrated that the phosphatase activity of SHP-2 is required for apoptosis and Bcl-x_L degradation, I hypothesized that SHP-2 was desphosphorylating nucleolin. Inhibition of SHP-2 activation by compound C inhibited the Ang II-induced down-regulation of Bcl-x_L 3'UTR. I also provide evidence that inhibition of SHP-2 phosphatase activity with compound C prior to Ang II treatment prevented the displacement of nucleolin from the Bcl-x_L 3'UTR. Based on the previous studies and my data, I concluded that that Ang II-mediated dephosphorylation of nucleolin plays an essential role in the degradation of Bcl-x_L

mRNA.

While I confirmed my hypothesis that SHP-2 is essential for Bcl-x_L mRNA destabilization, the evidence for SHP-2 directly dephosphorylating nucleolin is yet to be found. Determined by the NetPhos 2.0 program, nucleolin has one possible tyrosine phosphorylation site (Figure 11). This site remains a possibility for which the tyrosine phosphatase SHP-2 can dephosphorylate directly. There are no previous reports that SHP-2 can directly dephosphorylate nucleolin, but an indirect pathway can be suggested. One possible pathway in which the activation of SHP-2 may lead to the dephosphorylation of nucleolin is through a PI3K/Akt kinase mechanism. Nucleolin also contains 6 serine and 4 threonine possible phosphorylation sites. The PI3K/Akt signaling pathway is generally recognized for its ability to counteract cell stress responses that lead to cell cycle arrest or cell apoptosis; the serine/threonine kinase Akt is able to phosphorylate proteins to promote cell survival [549]. Various studies have shown PI3K/Akt to phosphorylate nucleolin and stabilize the mRNA [550-552]. However, a number of studies have demonstrated that SHP-2 can dephosphorylate Akt, leading to cellular apoptosis and cell cycle arrest [300, 553]. Therefore, one indirect mechanism by which SHP-2 can regulate Bcl-x_L mRNA is through the dephosphorylation and inactivation of Akt, which would prevent nucleolin phosphorylation. The lack of constant Akt phosphorylation will leave nucleolin susceptible to phosphatases to dephosphorylate the protein and causing nuclear localization. I have evidence showing that Ang II-induced nuclear translocation of nucleolin resulted in the decreased interaction between the protein and 3'UTR of Bcl-x_L mRNA, causing down-regulation of Bcl-x_L mRNA and protein. This shift resulted

```
Bos Taurus Nucleolin   Sequence

203
LSYSATEE TLQEVFEKATHIKVPQNQNGKSKG  YAFIEFA SFEDAKEALNSCNKREIEGRAIRLELQGPRG   SPNARSQPSK      80
TLFVKGL SED TTEE TLKESFDGSIRARIV  TDRETG SSKGFGFVDFN SEEDAKAAKEAMEDGEIDGNKVTLD  WAKPKGEGG     160
FGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGFGGRGGFRGGRG                                    240

........T.......................Y......S.............................S.........      80
.......S..T...T..............T.....SS.........S.............     .....................     160
.......................................                                          240

Phosphorylation sites predicted:         Ser: 6   Thr: 4   Tyr: 1
```

Serine predictions

Name	Pos	Context	Score	Pred
Sequence	2	---LSYSAT	0.050	.
Sequence	4	-LSYSATEE	0.099	.
Sequence	30	QNGKSKGYA	0.254	.
Sequence	40	IEFASFEDA	0.993	*S*
Sequence	50	EALNSCNKR	0.022	.
Sequence	71	GPRGSPNAR	0.556	*S*
Sequence	76	PNARSQPSK	0.119	.
Sequence	79	RSQPSKTLF	0.370	.
Sequence	88	VKGLSEDTT	0.947	*S*
Sequence	99	TLKESFDGS	0.426	.
Sequence	103	SFDGSIRAR	0.430	.
Sequence	116	RETGSSKGF	0.901	*S*
Sequence	117	ETGSSKGFG	0.950	*S*
Sequence	127	VDFNSEEDA	0.996	*S*

Threonine predictions

Name	Pos	Context	Score	Pred
Sequence	6	SYSATEETL	0.034	.
Sequence	9	ATEETLQEV	0.757	*T*
Sequence	18	FEKATHIKV	0.075	.
Sequence	81	QPSKTLFVK	0.223	.
Sequence	91	LSEDTTEET	0 .975	*T*
Sequence	92	SEDTTEETL	0.235	.
Sequence	95	TTEETLKES	0.978	*T*
Sequence	110	ARIVTDRET	0.987	*T*
Sequence	114	TDRETGSSK	0.183	.
Sequence	149	GNKVTLDWA	0.081	.

Tyrosine predictions

Name	Pos	Context	Score	Pred
Sequence	3	--LSYSATE	0.182	.
Sequence	33	KSKGYAFIE	0.522	*Y*

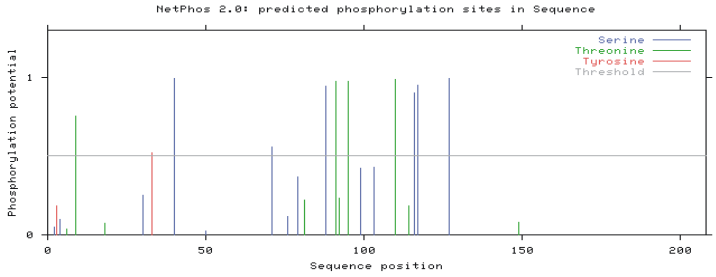


Figure 11: Predicted nucleolin phosphorylation sites by Netphos 2.0. Netphos 2.0 revealed 11 possible phosphorylation sites in Bos taurus nucleolin sequence. The predicted phosphorylation include 6 serine, 4 thereonine and 1 tyrosine sites.

in a decreased ratio of anti-apoptotic to pro-apoptotic which would be sufficient to induce apoptosis.

HGF inhibition of Ang II-induced mitochondrial-dependent apoptosis

HGF has been identified as an anti-apoptotic and anti-fibrotic factor which has been shown to prevent aberrant repair mechanisms that can lead to fibrotic remodeling in several organ systems, including in the lung, heart, kidney and liver [554-557]. HGF as a modulator of apoptosis is supported by numerous observations in different cell types [558-560]. HGF has shown the ability to upregulate anti-apoptotic Bcl-x_L mRNA and proteins to prevent cellular apoptosis [526, 561, 562]. In accordance with this, I have shown that HGF treatment upregulated Bcl-x_L mRNA and proteins in PAEC. Because the relative levels and ratio of anti-apoptotic proteins to pro-apoptotic proteins play a critical role in determining cell survival or death [563, 564], the upregulation of Bcl-x_L strongly suggests that HGF shifts PAEC towards cell survival and growth. Upon the elevated expression of Bcl-x_L, the protein can bind to Bax and neutralize all of its pro-apoptotic effects. To determine if this was true in my system, I co-immunoprecipated Bcl-x_L and Bax proteins. While the treatment of Ang II decreased the interaction between those two proteins, pretreatment with HGF resulted in continuous protein-protein interaction. This interaction between Bcl-x_L and Bax proteins prevents Bax dimerization and subsequent MOMP effects. This data indicates that HGF prevents Ang II-induced MOMP and the intrinsic apoptotic mechanism of cell death. I also provide evidence of HGF-mediated inhibition of Ang

II-induced DNA fragmentation and apoptosis. I have validated that, in both *in vitro* and *ex-vivo*, HGF prevents Ang II-induced cytochrome *c* release and the activation of caspase 3. From these data, I conclude that HGF prevents Ang II-induced mitochondrial-dependent apoptosis in PAEC.

HGF regulation of nucleolin

As Ang II mediates the nuclear localization of nucleolin and subsequent Bcl-x_L mRNA degradation and cellular apoptosis, I hypothesized that HGF will prevent the Ang II-mediated dephosphorylation of the nucleolin and nuclear localization. Due to the lack of specific phospho-nucleolin antibodies that can detect bovine protein, the detection of phosphorylated protein on a western blot could not be done. However, cell fractionation and immunofluoresence staining showed that while Ang II mediates nuclear localization of nucleolin, pretreating the cells with HGF prevented this nuclear translocation. The HGF-mediated prevention of the nuclear localization of nucleolin would allow binding and stabilization of cytoplasmic Bcl-x_L mRNA. RNA-immunoprecipitation of nucleolin demonstrated that HGF induces a constant interaction between the Bcl-x_L mRNA and nucleolin even after the Ang II treatment. From this, I concluded that HGF inhibits Ang II-induced apoptosis by inducing and keeping the interaction between the nucleolin and the 3'UTR of Bcl-x_L mRNA, thus stabilizing the anti-apoptotic Bcl-x_L mRNA and the protein.

I hypothesized that HGF-induced phosphorylation of nucleolin would cause cytosolic localization and allow the nucleolin-3'UTR mRNA binding and stabilize

Bcl-x_L mRNA. However, the mechanism by which HGF induced phosphorylation was unknown. There are two major pathways currently known for HGF-induced growth and inhibition of apoptosis: p42/p44 MAPK and PI3K/Akt pathways [565, 566]. HGF-mediated activation of the PI3K/Akt pathway is associated with cell motility [567-572] and cell survival [300, 553]. Akt has shown the ability to exert its anti-apoptotic effects in a variety of ways, including the phosphorylation of nucleolin [550-552]. Another mechanism whereby Akt inhibits apoptosis is through phosphorylation of the pro-apoptotic protein, Bad [573]. Dephosphorylated Bad forms a heterodimer with Bcl-x_L inactivating it and thus allowing Bax/Bak to initiate apoptosis [574]. However, Akt-phosphorylated Bad interacts with the 14-3-3 protein homodimer [574], which allows Bcl-2 proteins to bind and inhibit Bax, preventing cell death [574]. A previous study demonstrated that Akt can phosphorylate pro-caspase 9 and prevent the protease activity to suppress the activation of effector caspase 3 [575]. To determine whether HGF activates the PI3K/Akt pathway to phosphorylate nucleolin, I treated PAEC with the PI3K inhibitor LY294002 followed by HGF and Ang II treatments. If Akt was responsible for the phosphorylation of nucleolin, I would have expected LY294002 to inhibit the effect of HGF on the protein, and Ang II to dephosphorylate and translocate nucleolin to the nucleus. However, the inhibition of PI3K did not have significant effect on the localization of the nucleolin. This suggests that PI3K activity is not responsible for the HGF-mediated phosphorylation and subsequent cytoplasmic localization of nucleolin.

The cell proliferation and survival pathway by which HGF activates the cMet/MAPK cascade has been described in many normal and transformed cells [576-

579]. HGF has shown the ability to phosphorylate p42/p44 MAPK primarily through the Gab1/Grb2/Ras pathway [580-585]. Nakagami *et al.* [586] and Gao *et al.* [587] found that HGF-induced p42/p44 MAPK activation inhibited apoptosis induced by UV irradiation and chemotherapeutic drugs. Studies have demonstrated that elevated MAPK activity is associated with decreased caspase 3 activity and increased cell survival [588]. It has also been reported that MAPK may regulate the phosphorylation state of anti-apoptotic proteins [233, 589]. Using the specific MEK inhibitor U0126 as well as a dominant negative MEK-expressing adenovirus (adv DN MEK), I have demonstrated both *in vitro* and *ex-vivo* that HGF utilizes the MAPK pathway to protect PAEC from undergoing apoptosis. HGF pretreatment inhibited Ang II-induced caspase 3 activation. The addition of U0126 or adv DN MEK to the HGF and Ang II treatments resulted in an elevated level of cleaved caspase 3. This data suggest that MEK inhibition prevented p42/p44 MAPK from phosphorylating nucleolin, causing a displacement of nucleolin to the nucleus. I have also demonstrated that U0126 treatment prior to HGF and Ang II treatments resulted in nuclear nucleolin translocation, confirming that p42/p44 MAPK is necessary to phosphorylate nucleolin and localize it to the cytoplasm. HGF treatment prior to Ang II presented increased 3'UTR Bcl-x_L level compared to the sole Ang II treatment. However, when U0126 was added prior to HGF and Ang II, the MEK inhibitor negated the effect of HGF-mediated phosphorylation of p42/p44 MAPK, allowing Ang II to decrease the Bcl-x_L level.

It should be noted that in both *in vitro* and *ex-vivo* models, while the combined treatments of U0126 (or adv DN MEK), HGF, and Ang II significantly increased the

level of cleaved caspase 3 compared to HGF and Ang II treatment, it did not increase enough to equal the level of Ang II treatment alone. This suggests that HGF might activate another anti-apoptotic pathway, such as PI3K along with the MAPK, to mediate cell survival. To determine if this was the case, I treated the PAEC with LY294002, U0126, HGF, and Ang II. When U0126 and LY294002 were added together to inhibit both PI3K/Akt and p42/p44 MAPK, along with HGF and Ang II, the level of cleaved caspase 3 was similar to the Ang II treatment alone. This data demonstrates that both pathways need to be inhibited to fully negate the effects of HGF. Overall, the data suggests that while HGF primarily utilizes p42/p44 MAPK to inhibit Ang II-induced apoptosis, HGF also activates PI3K to elicit the anti-apoptotic mechanisms of the pathway. Because I demonstrated that PI3K does not have any effect on the nucleolin, I hypothesize that PI3K acts to phosphorylate Bad and help inhibit mitochondrial dysfunction together with the MAPK pathway.

Pulmonary fibrosis is a progressive disease resulting from the abnormal regeneration of cells after apoptosis. In the lungs, the air sacs and their supporting structures become inflamed and scarred. As the scarring continues, the lungs stiffen due to the deposition of collagens and other extracellular matrix proteins. The thickening and stiffing of the lung eventually leads to difficulty breathing and lack of oxygen in the bloodstream. Currently, there are no treatments for pulmonary fibrosis, other than lung transplantation. The identification of specific pathways and mechanisms for Ang II-induced apoptosis in fibrotic remodeling allows for comprehensive investigation into possible inhibitors of this process, such as HGF. The

known mechanism of HGF inhibition of Ang II-induced apoptosis may provide novel targets for the mitigation of fibrotic diseases. Here, I have identified a novel mechanism for Ang II-induced apoptosis that involves SHP-2-induced destabilization of Bcl-x_L mRNA through reduction of nucleolin binding (Figure 12). I have also demonstrated the ability of HGF to inhibit Ang II-induced apoptosis by stabilizing the Bcl-x_L mRNA through the phosphorylation and cytoplasmic localization of the nucleolin. Based on the work in this thesis, I believe that HGF offers an exciting potential therapy for pulmonary fibrosis.

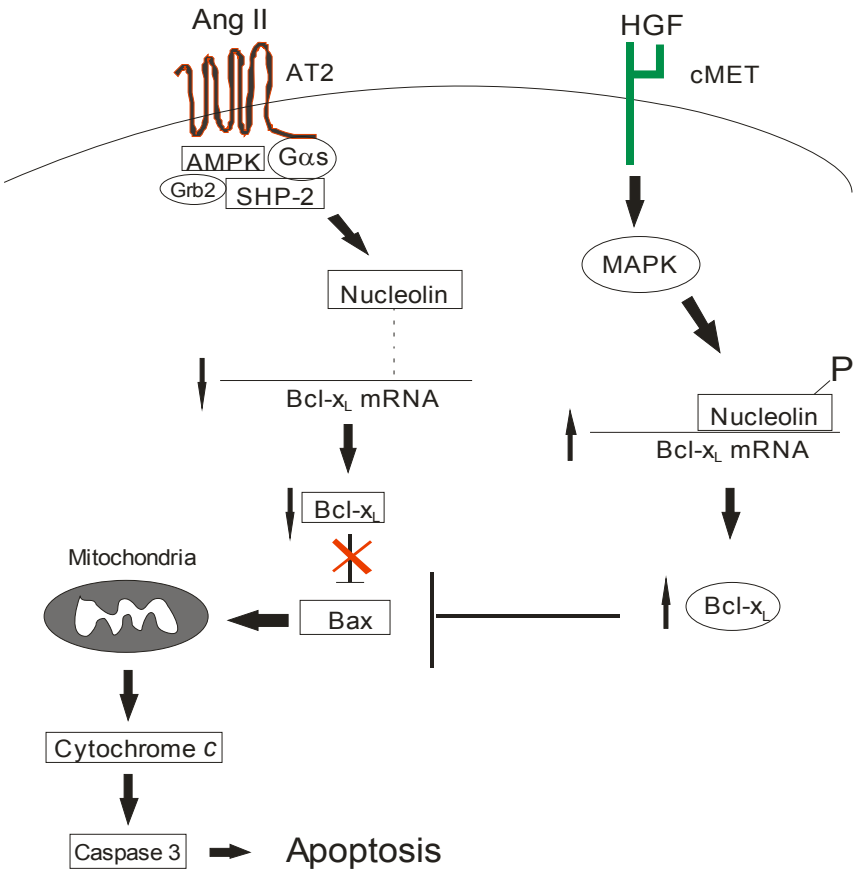


Figure 12: Mechanism of HGF inhibition of Ang II-induced apoptosis. Ang II bind to the AT2 receptor and activates SHP-2 via AMPK phosphorylation in the presence of the inactive Gαs subunit. SHP-2 then dephosphorylates and translocates the nucleolin protein to the nucleus, exposing the AU rich region of the Bcl-x $_L$mRNA, thus decreasing the mRNA decay. The decreased ratio of anti-apoptotic to pro-apoptotic leads to apoptosis by cytochrome $_c$ release from the mitochondria and the activation of caspase 3 protein. HGF binds to its cMET receptor, phosphorylating the MAPK protein. MAPK phosphorylates and localizes the nucleolin protein in the cytoplasm. Cytosolic nucleolin binds to the AU rich region of the Bcl-x$_L$ mRNA, stabilizing the mRNA. This shifts the balance towards the anti-apoptotic proteins, thus inhibiting the Ang II-induced apoptosis.

Chapter 7

Global Relevance and Long Term Goals

The long term goal of this study is to develop targeted therapy using HGF in IPF, with the principles that this molecule can be considered a biological response modifier to prevent and also to repress fibrosis in lung injuries by targeting relevant biomarkers. The objective of this thesis was to understand the signaling mechanism of Ang II-induced apoptosis and also to evaluate the cell protection and survival effect of HGF. The central hypothesis of the proposed study was lung fibrosis and IPF is mediated by Ang II and HGF can protect and repair the lung by interfering with the downstream signal transduction cascade of Ang II. The rationale of the proposed and future research is that the advanced comprehension of the signaling pathways that regulate lung epithelial/endothelial apoptosis and cell survival will lead to new strategies that can be used to prevent and/or treat lung injury and fibrosis, thereby reducing the morbidity and mortality that are associated with this condition.

The data from the current research show that HGF activates a MAPK-dependent, and not a PI3K-dependent phosphorylation of the nucleolin protein, which results in the stabilization of the Bcl-x_L mRNA, subsequently restoring the pro- and anti-apoptotic protein balance that was previously offset by Ang II activity. However, when the PAECs and *ex vivo* lung tissues were pretreated MEK inhibitor U0126 or dominant negative MEK-expressing adenovirus (Adv DN MEK) prior to HGF and by Ang II treatment, the level of cleaved caspase 3 was not restored to the level seen with the Ang II treatment alone. From the differences of expression level of the cleaved caspase 3 with HGF/U0126 or Adv DN MEK/Ang II treatment compared with that of Ang II alone, it can be suspected that HGF still has an anti-apoptotic effect, possibly through a PI3K pathway-mediated mechanism. HGF has been previously shown to activate the

PI3K/AKT pathway to inhibit apoptosis through phosphorylation and inactivation of the pro-apoptotic protein Bad [590] and also to increase Bcl-x_L [591]. . In order to fully understand the ability of HGF to rescue epithelial and endothelial cells from apoptosis during fibrosis, it will be important to investigate other possible signaling mechanisms by which HGF can inhibit Ang II-induced apoptosis.

Future directions of this study should involve the comprehensive investigation of the isoforms of HGF. While HGF has repeatedly shown to be an effective anti-apoptotic and anti-fibrotic agent, it has not been used effectively in a clinical setting. HGF contains an N-terminal hairpin loop and four kringle domains (K1, K2, K3, and K4). The structure is held together by 20 disulfide bonds. Due to the complex structure that is folded by the disulfide bonds, HGF cannot be reliably produced in mass amounts in the bacterial or in the yeast system. Thus, the problem of quantity of HGF in addition to the quality hinders the use of this molecule in the clinic. HGF also has naturally occurring isoforms due to alternative splicing at the kringle domains. NK2, N-terminal hairpin loop and two kringle domains (K1, K2), is a commonly studied isoform [565]. While the NK2 isoform is significantly shorter than the full length HGF protein with only 8 disulfide bonds, it is not commercially available, although the protein can be made in *E. coli* and refolded. While HGF has been shown to induce cell motility, growth and morphogenesis, the NK2 splice variant induces cell motility but not cell growth [565]. We have found that in normal lung epithelial and endothelial cells, HGF activates both the PI3K and p42/p44/MAPK pathways, while NK2 only activates the p42/p44/MAPK pathway (unpublished finding). The thesis work shown here and the unpublished preliminary findings results indicate that the

activation of the MAPK pathway is sufficient to induce a significant portion of the anti-apoptotic activity of HGF in lung epithelial and endothelial cell cultures.

While NK2 may be a clinically viable alternative to full-length HGF, NK2 produced in bacteria must still be denatured and refolded into the correct conformation. To overcome this obstacle, our lab has begun investigation into the use of the internalin B protein (InlB) of *Listeria monocytogenes* that was found to bind to and partially activate cMet. Studies have shown that the InlB protein functions like HGF by binding to the cMet, and activating p42/p44 MAPK and the PI3K/Akt pathway [592-596]. The structure of the InlB protein segment that binds to the cMet receptor was shown to have β-sheet conformation with no disulfide bonds and can be easily produced in standard *E. coli* (unpublished finding).

Our lab has invented a tandem dimer of the InlB protein which has shown to improve and strengthen the activation of both the PI3K/Akt and MAPK pathways (US Patent Serial no. 61/122,055). Our preliminary data has shown this dimer to be as effective as the HGF treatment in cell growth and motility. The tandem dimer of InlB inhibits both Ang II- and bleomycin-induced apoptosis in bovine PAEC, human bronchial epithelial cells as well as in the *ex vivo* rodent lungs. I believe it is crucial to continue studying the signaling mechanism of both HGF and InlB to determine its effectiveness in cell apoptosis inhibition. The ability of InlB to act as a HGF surrogate may prove to be an enormous leap towards the successful treatment of pulmonary fibrosis diseases.

Chapter 8

References

1. Uhal, B.D., et al., *Angiotensin-TGF-beta 1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages.* Curr Pharm Des, 2007. 13(12): p. 1247-56.
2. Hashimoto, S., et al., *Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome.* Am J Respir Crit Care Med, 2000. 161(1): p. 237-43.
3. Matute-Bello, G., et al., *Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS).* J Immunol, 1999. 163(4): p. 2217-25.
4. Albertine, K.H., et al., *Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome.* Am J Pathol, 2002. 161(5): p. 1783-96.
5. Hyers, T.M., et al., *Tumor necrosis factor levels in serum and bronchoalveolar lavage fluid of patients with the adult respiratory distress syndrome.* Am Rev Respir Dis, 1991. 144(2): p. 268-71.
6. Hagimoto, N., et al., *[DNA strand breaks in epithelial cells from mice with bleomycin induced pulmonary fibrosis].* Nihon Kyobu Shikkan Gakkai Zasshi, 1996. 34(1): p. 3-8.
7. Kuwano, K., et al., *P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 1996. 154(2 Pt 1): p. 477-83.
8. Uhal, B.D., et al., *Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung.* Am J Physiol, 1998. 275(6 Pt 1): p. L1192-9.
9. Cooper, J.A., Jr., *Pulmonary fibrosis: pathways are slowly coming into light.* Am J Respir Cell Mol Biol, 2000. 22(5): p. 520-3.
10. Fujita, M., et al., *Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice.* Int Arch Allergy Immunol, 1998. 117(3): p. 202-8.
11. Kawasaki, M., et al., *Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor.* Am J Pathol, 2000. 157(2): p. 597-603.
12. Hagimoto, N., et al., *Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice.* Am J Respir Cell Mol Biol, 1997. 16(1): p. 91-101.
13. Uhal, B.D., *Epithelial apoptosis in the initiation of lung fibrosis.* Eur Respir J Suppl, 2003. 44: p. 7s-9s.
14. Kuwano, K., et al., *Expression of apoptosis-regulatory genes in epithelial cells in pulmonary fibrosis in mice.* J Pathol, 2000. 190(2): p. 221-9.
15. Hagimoto, N., et al., *Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen.* Am J Respir Cell Mol Biol, 1997. 17(3): p. 272-8.
16. Uhal, B.D., et al., *Inhibition of amiodarone-induced lung fibrosis but not alveolitis by angiotensin system antagonists.* Pharmacol Toxicol, 2003. 92(2): p. 81-7.
17. Wang, R., et al., *Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor.* Am J Physiol Lung Cell Mol Physiol, 2000. 279(1): p. L143-51.

18. Uhal, B.D., *Apoptosis in lung fibrosis and repair.* Chest, 2002. 122(6 Suppl): p. 293S-298S.
19. Bradley, K.H., S.D. McConnell, and R.G. Crystal, *Lung collagen composition and synthesis. Characterization and changes with age.* J Biol Chem, 1974. 249(9): p. 2674-83.
20. Hayek, H.v., *The human lung*. 1960, New York,: Hafner Pub. Co. 372 p.
21. Pierce, J.A. and J.B. Hocott, *Studies on the collagen and elastin content of the human lung.* J Clin Invest, 1960. 39: p. 8-14.
22. Fitzpatrick, M. and V.D. Hospelhorn, *Studies of human pulmonary connective tissue. II. Amino acid composition of residues following collagenase digestion of lung connective tissues.* Am Rev Respir Dis, 1965. 92(5): p. 792-800.
23. Kefalides, N.A. and B. Denduchis, *Structural components of epithelial and endothelial basement membranes.* Biochemistry, 1969. 8(11): p. 4613-21.
24. John, R. and J. Thomas, *Chemical compositions of elastins isolated from aortas and pulmonary tissues of humans of different ages.* Biochem J, 1972. 127(1): p. 261-9.
25. Pierce, R.J., et al., *Estimation of lung volumes from chest radiographs using shape information.* Thorax, 1979. 34(6): p. 726-34.
26. Low, F.N., *Extracellular components of the pulmonary alveolar wall.* Arch Intern Med, 1971. 127(5): p. 847-52.
27. Nagaishi, C.u.o. and N. Nagasawa, *Functional anatomy and histology of the lung*. [American ed. 1972, Baltimore,: University Park Press. xxviii, 295 p.
28. Nixon, W. and A. Pack, *Effect of altered gas diffusivity on alveolar gas exchange-a theoretical study.* J Appl Physiol, 1980. 48(1): p. 147-53.
29. Schwarz, M.I. and T.E. King, *Interstitial lung disease*. 1988, Toronto ; Philadelphia: B.C. Decker. 350 p.
30. Campbell, E.J., R.M. Senior, and H.G. Welgus, *Extracellular matrix injury during lung inflammation.* Chest, 1987. 92(1): p. 161-7.
31. McDonald, J.A., et al., *A monoclonal antibody to the carboxyterminal domain of procollagen type I visualizes collagen-synthesizing fibroblasts. Detection of an altered fibroblast phenotype in lungs of patients with pulmonary fibrosis.* J Clin Invest, 1986. 78(5): p. 1237-44.
32. Roman, J., *Extracellular matrix and lung inflammation.* Immunol Res, 1996. 15(2): p. 163-78.
33. Gudewicz, P.W., et al., *Priming of human monocyte superoxide production and arachidonic acid metabolism by adherence to collagen- and basement membrane-coated surfaces.* J Leukoc Biol, 1994. 55(4): p. 423-9.
34. Pacifici, R., et al., *Collagen-induced release of interleukin 1 from human blood mononuclear cells. Potentiation by fibronectin binding to the alpha 5 beta 1 integrin.* J Clin Invest, 1992. 89(1): p. 61-7.
35. Ruoslahti, E. and J.C. Reed, *Anchorage dependence, integrins, and apoptosis.* Cell, 1994. 77(4): p. 477-8.
36. Khalil, N. and R. O'Connor, *Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment.* Cmaj, 2004. 171(2): p. 153-60.

37. Pardo, A. and M. Selman, *Molecular mechanisms of pulmonary fibrosis.* Front Biosci, 2002. 7: p. d1743-61.
38. Selman, M. and A. Pardo, *Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder.* Respir Res, 2002. 3: p. 3.
39. Gross, T.J. and G.W. Hunninghake, *Idiopathic pulmonary fibrosis.* N Engl J Med, 2001. 345(7): p. 517-25.
40. Pober, J.S. and R.S. Cotran, *The role of endothelial cells in inflammation.* Transplantation, 1990. 50(4): p. 537-44.
41. *American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS).* Am J Respir Crit Care Med, 2000. 161(2 Pt 1): p. 646-64.
42. ATS, *American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS).* Am J Respir Crit Care Med, 2000. 161(2 Pt 1): p. 646-64.
43. Harari, S. and A. Caminati, *Idiopathic pulmonary fibrosis.* Allergy, 2005. 60(4): p. 421-35.
44. Chapman, H.A., *Disorders of lung matrix remodeling.* J Clin Invest, 2004. 113(2): p. 148-57.
45. Iwai, K., et al., *Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure.* Am J Respir Crit Care Med, 1994. 150(3): p. 670-5.
46. Hubbard, R., et al., *Occupational exposure to metal or wood dust and aetiology of cryptogenic fibrosing alveolitis.* Lancet, 1996. 347(8997): p. 284-9.
47. Baumgartner, K.B., et al., *Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 1997. 155(1): p. 242-8.
48. Hubbard, R., et al., *Exposure to commonly prescribed drugs and the etiology of cryptogenic fibrosing alveolitis: a case-control study.* Am J Respir Crit Care Med, 1998. 157(3 Pt 1): p. 743-7.
49. Scott, J., I. Johnston, and J. Britton, *What causes cryptogenic fibrosing alveolitis? A case-control study of environmental exposure to dust.* Bmj, 1990. 301(6759): p. 1015-7.
50. Baumgartner, K.B., et al., *Occupational and environmental risk factors for idiopathic pulmonary fibrosis: a multicenter case-control study. Collaborating Centers.* Am J Epidemiol, 2000. 152(4): p. 307-15.
51. Billings, C.G. and P. Howard, *Hypothesis: exposure to solvents may cause fibrosing alveolitis.* Eur Respir J, 1994. 7(6): p. 1172-6.
52. Marsh, P., I. Johnston, and J. Britton, *Atopy as a risk factor for cryptogenic fibrosing alveolitis.* Respir Med, 1994. 88(5): p. 369-71.
53. Vergnon, J.M., et al., *Cryptogenic fibrosing alveolitis and Epstein-Barr virus: an association?* Lancet, 1984. 2(8406): p. 768-71.
54. Egan, J.J., et al., *Epstein-Barr virus replication within pulmonary epithelial cells in cryptogenic fibrosing alveolitis.* Thorax, 1995. 50(12): p. 1234-9.
55. Watanabe, A., et al., *[A case of Epstein-Barr virus infection with interstitial pneumonitis].* Nihon Kyobu Shikkan Gakkai Zasshi, 1987. 25(7): p. 794-8.

56. Meliconi, R., et al., *Incidence of hepatitis C virus infection in Italian patients with idiopathic pulmonary fibrosis.* Thorax, 1996. 51(3): p. 315-7.
57. Irving, W.L., S. Day, and I.D. Johnston, *Idiopathic pulmonary fibrosis and hepatitis C virus infection.* Am Rev Respir Dis, 1993. 148(6 Pt 1): p. 1683-4.
58. Ueda, T., et al., *Idiopathic pulmonary fibrosis and high prevalence of serum antibodies to hepatitis C virus.* Am Rev Respir Dis, 1992. 146(1): p. 266-8.
59. Pinsker, K.L., et al., *Usual interstitial pneumonia following Texas A2 influenza infection.* Chest, 1981. 80(2): p. 123-6.
60. Louria, D.B., et al., *Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza.* J Clin Invest, 1959. 38(1 Part 2): p. 213-65.
61. Jakab, G.J. and D.J. Bassett, *Influenza virus infection, ozone exposure, and fibrogenesis.* Am Rev Respir Dis, 1990. 141(5 Pt 1): p. 1307-15.
62. Jiwa, M., et al., *Three sensitive methods for the detection of cytomegalovirus in lung tissue of patients with interstitial pneumonitis.* Am J Clin Pathol, 1990. 93(4): p. 491-4.
63. Jakab, G.J., *Sequential virus infections, bacterial superinfections, and fibrogenesis.* Am Rev Respir Dis, 1990. 142(2): p. 374-9.
64. Hartelius, H., et al., *Computed tomography of the lungs in acquired immunodeficiency syndrome. An early indicator of interstitial pneumonia.* Acta Radiol, 1988. 29(6): p. 641-4.
65. Siegel, C., S. Johnston, and S. Adair, *Isolation of measles virus in primary rhesus monkey cells from a child with acute interstitial pneumonia who cytologically had giant-cell pneumonia without a rash.* Am J Clin Pathol, 1990. 94(4): p. 464-9.
66. Weintrub, P.S., et al., *Giant cell pneumonia caused by parainfluenza type 3 in a patient with acute myelomonocytic leukemia.* Arch Pathol Lab Med, 1987. 111(6): p. 569-70.
67. Carrigan, D.R., et al., *Interstitial pneumonitis associated with human herpesvirus-6 infection after marrow transplantation.* Lancet, 1991. 338(8760): p. 147-9.
68. Kaufman, J.M., C.A. Cuvelier, and M. Van der Straeten, *Mycoplasma pneumonia with fulminant evolution into diffuse interstitial fibrosis.* Thorax, 1980. 35(2): p. 140-4.
69. Chastre, J., et al., *Pulmonary fibrosis following pneumonia due to acute Legionnaires' disease. Clinical, ultrastructural, and immunofluorescent study.* Chest, 1987. 91(1): p. 57-62.
70. Ellis, R.H., *Familial Incidence of Diffuse Interstitial Pulmonary Fibrosis.* Postgrad Med J, 1965. 41: p. 150-2.
71. Murphy, A. and B.J. O'Sullivan, *Familial fibrosing alveolitis.* Ir J Med Sci, 1981. 150(7): p. 204-9.
72. Bitterman, P.B., et al., *Familial idiopathic pulmonary fibrosis. Evidence of lung inflammation in unaffected family members.* N Engl J Med, 1986. 314(21): p. 1343-7.
73. Mageto, Y.N. and G. Raghu, *Genetic predisposition of idiopathic pulmonary fibrosis.* Curr Opin Pulm Med, 1997. 3(5): p. 336-40.

74. Wolf, G., *Vasoactive substances as regulators of renal growth.* Exp Nephrol, 1993. 1(3): p. 141-51.
75. Wang, R., et al., *Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides.* Am J Physiol, 1999. 277(6 Pt 1): p. L1158-64.
76. Kawano, H., et al., *Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts.* Circulation, 2000. 101(10): p. 1130-7.
77. Marshall, R.P., R.J. McAnulty, and G.J. Laurent, *Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor.* Am J Respir Crit Care Med, 2000. 161(6): p. 1999-2004.
78. Wagner, G.R., M.D. Attfield, and J.E. Parker, *Chest radiography in dust-exposed miners: promise and problems, potential and imperfections.* Occup Med, 1993. 8(1): p. 127-41.
79. Coultas, D.B., et al., *The epidemiology of interstitial lung diseases.* Am J Respir Crit Care Med, 1994. 150(4): p. 967-72.
80. Panos, R.J., et al., *Clinical deterioration in patients with idiopathic pulmonary fibrosis: causes and assessment.* Am J Med, 1990. 88(4): p. 396-404.
81. Schwartz, D.A., et al., *Determinants of survival in idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 1994. 149(2 Pt 1): p. 450-4.
82. Coultas, D.B. and M.P. Hughes, *Accuracy of mortality data for interstitial lung diseases in New Mexico, USA.* Thorax, 1996. 51(7): p. 717-20.
83. Johnston, I.D., et al., *British Thoracic Society study of cryptogenic fibrosing alveolitis: current presentation and initial management. Fibrosing Alveolitis Subcommittee of the Research Committee of the British Thoracic Society.* Thorax, 1997. 52(1): p. 38-44.
84. Gay, S.E., et al., *Idiopathic pulmonary fibrosis: predicting response to therapy and survival.* Am J Respir Crit Care Med, 1998. 157(4 Pt 1): p. 1063-72.
85. Hubbard, R., I. Johnston, and J. Britton, *Survival in patients with cryptogenic fibrosing alveolitis: a population-based cohort study.* Chest, 1998. 113(2): p. 396-400.
86. Mapel, D.W., et al., *Idiopathic pulmonary fibrosis: survival in population based and hospital based cohorts.* Thorax, 1998. 53(6): p. 469-76.
87. Strieter, R.M., *Mechanisms of pulmonary fibrosis: conference summary.* Chest, 2001. 120(1 Suppl): p. 77S-85S.
88. McAnulty, R.J. and G.J. Laurent, *Pathogenesis of lung fibrosis and potential new therapeutic strategies.* Exp Nephrol, 1995. 3(2): p. 96-107.
89. Egan, J.J. and A.A. Woodcock, *Does the treatment of cryptogenic fibrosing alveolitis influence prognosis?* Respir Med, 1996. 90(3): p. 127-30.
90. Rudd, R.M., P.L. Haslam, and M. Turner-Warwick, *Cryptogenic fibrosing alveolitis. Relationships of pulmonary physiology and bronchoalveolar lavage to response to treatment and prognosis.* Am Rev Respir Dis, 1981. 124(1): p. 1-8.
91. Johnston, I.D., et al., *The management of cryptogenic fibrosing alveolitis in three regions of the United Kingdom.* Eur Respir J, 1993. 6(6): p. 891-3.
92. Meier-Sydow, J., et al., *[Long-term follow-up of lung function parameters in patients with idiopathic pulmonary fibrosis treated with prednisone and*

azathioprin or d-penicillamine (author's transl)]. Prax Klin Pneumol, 1979. 33 Suppl 1: p. 680-8.
93. Raghu, G., et al., *Azathioprine combined with prednisone in the treatment of idiopathic pulmonary fibrosis: a prospective double-blind, randomized, placebo-controlled clinical trial.* Am Rev Respir Dis, 1991. 144(2): p. 291-6.
94. Winterbauer, R.H., et al., *Diffuse interstitial pneumonitis. Clinicopathologic correlations in 20 patients treated with prednisone/azathioprine.* Am J Med, 1978. 65(4): p. 661-72.
95. Johnson, M.A., et al., *Randomised controlled trial comparing prednisolone alone with cyclophosphamide and low dose prednisolone in combination in cryptogenic fibrosing alveolitis.* Thorax, 1989. 44(4): p. 280-8.
96. Peters, S.G., et al., *Colchicine in the treatment of pulmonary fibrosis.* Chest, 1993. 103(1): p. 101-4.
97. Hubbard, R., et al., *Mortality rates from cryptogenic fibrosing alveolitis in seven countries.* Thorax, 1996. 51(7): p. 711-6.
98. Strieter, R.M., *Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel.* Chest, 2005. 128(5 Suppl 1): p. 526S-532S.
99. Uhal, B.D., et al., *Fibroblasts isolated after fibrotic lung injury induce apoptosis of alveolar epithelial cells in vitro.* Am J Physiol, 1995. 269(6 Pt 1): p. L819-28.
100. Geiser, T., *Idiopathic pulmonary fibrosis--a disorder of alveolar wound repair?* Swiss Med Wkly, 2003. 133(29-30): p. 405-11.
101. Selman, M., T.E. King, and A. Pardo, *Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy.* Ann Intern Med, 2001. 134(2): p. 136-51.
102. Pardo, A. and M. Selman, *Idiopathic pulmonary fibrosis: new insights in its pathogenesis.* Int J Biochem Cell Biol, 2002. 34(12): p. 1534-8.
103. White, E.S., M.H. Lazar, and V.J. Thannickal, *Pathogenetic mechanisms in usual interstitial pneumonia/idiopathic pulmonary fibrosis.* J Pathol, 2003. 201(3): p. 343-54.
104. Phan, S.H., *The myofibroblast in pulmonary fibrosis.* Chest, 2002. 122(6 Suppl): p. 286S-289S.
105. Gabbiani, G., *The myofibroblast in wound healing and fibrocontractive diseases.* J Pathol, 2003. 200(4): p. 500-3.
106. Kuhn, C., 3rd, et al., *An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis.* Am Rev Respir Dis, 1989. 140(6): p. 1693-703.
107. Kuhn, C. and J.A. McDonald, *The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis.* Am J Pathol, 1991. 138(5): p. 1257-65.
108. Fukuda, Y., et al., *Significance of early intra-alveolar fibrotic lesions and integrin expression in lung biopsy specimens from patients with idiopathic pulmonary fibrosis.* Hum Pathol, 1995. 26(1): p. 53-61.
109. Katzenstein, A.L. and J.L. Myers, *Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification.* Am J Respir Crit Care Med, 1998. 157(4 Pt 1): p. 1301-15.

110. Iwano, M., et al., *Evidence that fibroblasts derive from epithelium during tissue fibrosis.* J Clin Invest, 2002. 110(3): p. 341-50.
111. Kim, K.K., et al., *Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix.* Proc Natl Acad Sci U S A, 2006. 103(35): p. 13180-5.
112. Willis, B.C., R.M. duBois, and Z. Borok, *Epithelial origin of myofibroblasts during fibrosis in the lung.* Proc Am Thorac Soc, 2006. 3(4): p. 377-82.
113. Hashimoto, N., et al., *Bone marrow-derived progenitor cells in pulmonary fibrosis.* J Clin Invest, 2004. 113(2): p. 243-52.
114. Quan, T.E., et al., *Circulating fibrocytes: collagen-secreting cells of the peripheral blood.* Int J Biochem Cell Biol, 2004. 36(4): p. 598-606.
115. Dunsmore, S.E. and S.D. Shapiro, *The bone marrow leaves its scar: new concepts in pulmonary fibrosis.* J Clin Invest, 2004. 113(2): p. 180-2.
116. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. 26(4): p. 239-57.
117. Prindull, G., *Apoptosis in the embryo and tumorigenesis.* Eur J Cancer, 1995. 31A(1): p. 116-23.
118. Meier, P., A. Finch, and G. Evan, *Apoptosis in development.* Nature, 2000. 407(6805): p. 796-801.
119. Thompson, C.B., *Apoptosis in the pathogenesis and treatment of disease.* Science, 1995. 267(5203): p. 1456-62.
120. Savill, J. and V. Fadok, *Corpse clearance defines the meaning of cell death.* Nature, 2000. 407(6805): p. 784-8.
121. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. 407(6805): p. 770-6.
122. Leist, M. and M. Jaattela, *Four deaths and a funeral: from caspases to alternative mechanisms.* Nat Rev Mol Cell Biol, 2001. 2(8): p. 589-98.
123. Sartorius, U., I. Schmitz, and P.H. Krammer, *Molecular mechanisms of death-receptor-mediated apoptosis.* Chembiochem, 2001. 2(1): p. 20-9.
124. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation.* Science, 1998. 281(5381): p. 1305-8.
125. Green, D.R., et al., *Ten years of publication in cell death.* Cell Death Differ, 2004. 11(1): p. 2-3.
126. Shi, Y., *Mechanisms of caspase activation and inhibition during apoptosis.* Mol Cell, 2002. 9(3): p. 459-70.
127. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. 10(8): p. 789-99.
128. Zhang, L., et al., *Role of BAX in the apoptotic response to anticancer agents.* Science, 2000. 290(5493): p. 989-92.
129. Green, D.R. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. 305(5684): p. 626-9.
130. Fesik, S.W., *Insights into programmed cell death through structural biology.* Cell, 2000. 103(2): p. 273-82.
131. Wilson, M.E., et al., *Immune modulation by estrogens: role in CNS HIV-1 infection.* Endocrine, 2006. 29(2): p. 289-97.

132. Grad, J.M., X.R. Zeng, and L.H. Boise, *Regulation of Bcl-xL: a little bit of this and a little bit of STAT.* Curr Opin Oncol, 2000. 12(6): p. 543-9.
133. Kim, M., et al., *Detection of Bax protein conformational change using a surface plasmon resonance imaging-based antibody chip.* Biochem Biophys Res Commun, 2005. 338(4): p. 1834-8.
134. Kirkin, V., S. Joos, and M. Zornig, *The role of Bcl-2 family members in tumorigenesis.* Biochim Biophys Acta, 2004. 1644(2-3): p. 229-49.
135. Mihara, M., et al., *p53 has a direct apoptogenic role at the mitochondria.* Mol Cell, 2003. 11(3): p. 577-90.
136. Chipuk, J.E., et al., *Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis.* Science, 2004. 303(5660): p. 1010-4.
137. Moll, U.M., et al., *Transcription-independent pro-apoptotic functions of p53.* Curr Opin Cell Biol, 2005. 17(6): p. 631-6.
138. Pecci, A., et al., *Promoter choice influences alternative splicing and determines the balance of isoforms expressed from the mouse bcl-X gene.* J Biol Chem, 2001. 276(24): p. 21062-9.
139. Bachelor, M.A. and G.T. Bowden, *Ultraviolet A-induced modulation of Bcl-XL by p38 MAPK in human keratinocytes: post-transcriptional regulation through the 3'-untranslated region.* J Biol Chem, 2004. 279(41): p. 42658-68.
140. Wu, L.X., et al., *CD28 regulates the translation of Bcl-xL via the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway.* J Immunol, 2005. 174(1): p. 180-94.
141. Zhang, J., G. Tsaprailis, and G.T. Bowden, *Nucleolin stabilizes Bcl-X L messenger RNA in response to UVA irradiation.* Cancer Res, 2008. 68(4): p. 1046-54.
142. Fadeel, B., S. Orrenius, and B. Zhivotovsky, *The most unkindest cut of all: on the multiple roles of mammalian caspases.* Leukemia, 2000. 14(8): p. 1514-25.
143. Cohen, G.M., *Caspases: the executioners of apoptosis.* Biochem J, 1997. 326 (Pt 1): p. 1-16.
144. Kurokawa, M. and S. Kornbluth, *Caspases and kinases in a death grip.* Cell, 2009. 138(5): p. 838-54.
145. Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri, *CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme.* J Biol Chem, 1994. 269(49): p. 30761-4.
146. Degterev, A., M. Boyce, and J. Yuan, *A decade of caspases.* Oncogene, 2003. 22(53): p. 8543-67.
147. Boatright, K.M., et al., *A unified model for apical caspase activation.* Mol Cell, 2003. 11(2): p. 529-41.
148. Donepudi, M., et al., *Insights into the regulatory mechanism for caspase-8 activation.* Mol Cell, 2003. 11(2): p. 543-9.
149. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. 281(5381): p. 1312-6.

150. Varfolomeev, E.E., et al., *Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally.* Immunity, 1998. 9(2): p. 267-76.
151. Kuida, K., et al., *Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9.* Cell, 1998. 94(3): p. 325-37.
152. Hakem, R., et al., *Differential requirement for caspase 9 in apoptotic pathways in vivo.* Cell, 1998. 94(3): p. 339-52.
153. Thornberry, N.A., et al., *A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis.* J Biol Chem, 1997. 272(29): p. 17907-11.
154. Slee, E.A., et al., *Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner.* J Cell Biol, 1999. 144(2): p. 281-92.
155. Woo, M., et al., *Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes.* Genes Dev, 1998. 12(6): p. 806-19.
156. Kuida, K., et al., *Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice.* Nature, 1996. 384(6607): p. 368-72.
157. Polunovsky, V.A., et al., *Role of mesenchymal cell death in lung remodeling after injury.* J Clin Invest, 1993. 92(1): p. 388-97.
158. Thannickal, V.J., et al., *Mechanisms of pulmonary fibrosis.* Annu Rev Med, 2004. 55: p. 395-417.
159. Crystal, R.G., *The lung : scientific foundations*. 2nd ed. 1997, Philadelphia: Lippincott-Raven. 2 v. (xlviii, 2811, 68 p.).
160. Leslie, C.C., et al., *Heparin-binding EGF-like growth factor is a mitogen for rat alveolar type II cells.* Am J Respir Cell Mol Biol, 1997. 16(4): p. 379-87.
161. Barbas-Filho, J.V., et al., *Evidence of type II pneumocyte apoptosis in the pathogenesis of idiopathic pulmonary fibrosis (IFP)/usual interstitial pneumonia (UIP).* J Clin Pathol, 2001. 54(2): p. 132-8.
162. Plataki, M., et al., *Expression of apoptotic and antiapoptotic markers in epithelial cells in idiopathic pulmonary fibrosis.* Chest, 2005. 127(1): p. 266-74.
163. Lee, C.G., et al., *Early growth response gene 1-mediated apoptosis is essential for transforming growth factor beta1-induced pulmonary fibrosis.* J Exp Med, 2004. 200(3): p. 377-89.
164. Wallach-Dayan, S.B., et al., *Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway.* Am J Physiol Lung Cell Mol Physiol, 2006. 290(4): p. L790-L796.
165. Kuwano, K., et al., *Mitochondria-mediated apoptosis of lung epithelial cells in idiopathic interstitial pneumonias.* Lab Invest, 2002. 82(12): p. 1695-706.
166. Kuwano, K., et al., *The involvement of Fas-Fas ligand pathway in fibrosing lung diseases.* Am J Respir Cell Mol Biol, 1999. 20(1): p. 53-60.
167. Kuwano, K., et al., *Increased circulating levels of soluble Fas ligand are correlated with disease activity in patients with fibrosing lung diseases.* Respirology, 2002. 7(1): p. 15-21.

168. Maeyama, T., et al., *Upregulation of Fas-signalling molecules in lung epithelial cells from patients with idiopathic pulmonary fibrosis.* Eur Respir J, 2001. 17(2): p. 180-9.
169. Cantin, A.M., et al., *Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis.* J Clin Invest, 1987. 79(6): p. 1665-73.
170. Cantin, A.M., R.C. Hubbard, and R.G. Crystal, *Glutathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis.* Am Rev Respir Dis, 1989. 139(2): p. 370-2.
171. Strausz, J., et al., *Oxygen radical production by alveolar inflammatory cells in idiopathic pulmonary fibrosis.* Am Rev Respir Dis, 1990. 141(1): p. 124-8.
172. Waghray, M., et al., *Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts.* Faseb J, 2005. 19(7): p. 854-6.
173. Hagimoto, N., et al., *TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells.* J Immunol, 2002. 168(12): p. 6470-8.
174. Desmouliere, A., et al., *Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar.* Am J Pathol, 1995. 146(1): p. 56-66.
175. Denton, C.P., et al., *Activation of a fibroblast-specific enhancer of the proalpha2(I) collagen gene in tight-skin mice.* Arthritis Rheum, 2001. 44(3): p. 712-22.
176. Dimmeler, S., et al., *Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide.* Circ Res, 1997. 81(6): p. 970-6.
177. Kisseleva, T. and D.A. Brenner, *Mechanisms of fibrogenesis.* Exp Biol Med (Maywood), 2008. 233(2): p. 109-22.
178. Zhang, H., et al., *Inhibitor of differentiation 1 promotes endothelial survival in a bleomycin model of lung injury in mice.* Am J Pathol, 2007. 171(4): p. 1113-26.
179. Houck, K.A., et al., *The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA.* Mol Endocrinol, 1991. 5(12): p. 1806-14.
180. Farkas, L., et al., *VEGF ameliorates pulmonary hypertension through inhibition of endothelial apoptosis in experimental lung fibrosis in rats.* J Clin Invest, 2009. 119(5): p. 1298-311.
181. Yoshida, K., et al., *MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis.* J Pathol, 2002. 198(3): p. 388-96.
182. Wendt, C.H., et al., *Alveolar epithelial cells regulate the induction of endothelial cell apoptosis.* Am J Physiol, 1994. 267(4 Pt 1): p. C893-900.
183. Lambert, D.W., N.M. Hooper, and A.J. Turner, *Angiotensin-converting enzyme 2 and new insights into the renin-angiotensin system.* Biochem Pharmacol, 2008. 75(4): p. 781-6.
184. Clauser, E., et al., *Angiotensin II receptors: protein and gene structures, expression and potential pathological involvements.* Eur J Endocrinol, 1996. 134(4): p. 403-11.
185. Timmermans, P.B., et al., *Angiotensin II receptors and angiotensin II receptor antagonists.* Pharmacol Rev, 1993. 45(2): p. 205-51.

186. Ardaillou, R., et al., *Biology and functions of renal receptors for angiotensin II and its active fragments.* Adv Nephrol Necker Hosp, 1998. 28: p. 225-57.
187. Leri, A., et al., *Up-regulation of AT(1) and AT(2) receptors in postinfarcted hypertrophied myocytes and stretch-mediated apoptotic cell death.* Am J Pathol, 2000. 156(5): p. 1663-72.
188. Jalowy, A., et al., *Infarct size reduction by AT1-receptor blockade through a signal cascade of AT2-receptor activation, bradykinin and prostaglandins in pigs.* J Am Coll Cardiol, 1998. 32(6): p. 1787-96.
189. Kajstura, J., et al., *Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats.* Am J Physiol, 1996. 271(3 Pt 2): p. H1215-28.
190. Liu, J.J., et al., *Increased apoptosis in the heart of genetic hypertension, associated with increased fibroblasts.* Cardiovasc Res, 2000. 45(3): p. 729-35.
191. Ishidoya, S., et al., *Angiotensin II receptor antagonist ameliorates renal tubulointerstitial fibrosis caused by unilateral ureteral obstruction.* Kidney Int, 1995. 47(5): p. 1285-94.
192. Truong, L.D., et al., *Cell apoptosis and proliferation in experimental chronic obstructive uropathy.* Kidney Int, 1996. 50(1): p. 200-7.
193. Morrissey, J.J. and S. Klahr, *Rapid communication. Enalapril decreases nuclear factor kappa B activation in the kidney with ureteral obstruction.* Kidney Int, 1997. 52(4): p. 926-33.
194. Kajstura, J., et al., *Angiotensin II induces apoptosis of adult ventricular myocytes in vitro.* J Mol Cell Cardiol, 1997. 29(3): p. 859-70.
195. Cigola, E., et al., *Angiotensin II activates programmed myocyte cell death in vitro.* Exp Cell Res, 1997. 231(2): p. 363-71.
196. Marshall, R.P., et al., *Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome.* Am J Respir Crit Care Med, 2002. 166(5): p. 646-50.
197. Morrison, C.D., et al., *Increased D allele frequency of the angiotensin-converting enzyme gene in pulmonary fibrosis.* Hum Pathol, 2001. 32(5): p. 521-8.
198. Hilgenfeldt, U., et al., *Renin-angiotensin system in sepsis.* Clin Exp Hypertens A, 1987. 9(8-9): p. 1493-504.
199. Konigshoff, M., et al., *The angiotensin II receptor 2 is expressed and mediates angiotensin II signaling in lung fibrosis.* Am J Respir Cell Mol Biol, 2007. 37(6): p. 640-50.
200. Marshall, R.P., *The pulmonary renin-angiotensin system.* Curr Pharm Des, 2003. 9(9): p. 715-22.
201. Komeda, K., et al., *Significance of chymase-dependent angiotensin II formation in the progression of human liver fibrosis.* Hepatol Res, 2007.
202. Socha, M.J., et al., *Secreted protein acidic and rich in cysteine deficiency ameliorates renal inflammation and fibrosis in angiotensin hypertension.* Am J Pathol, 2007. 171(4): p. 1104-12.
203. Bullock, G.R., et al., *Distribution of type-1 and type-2 angiotensin receptors in the normal human lung and in lungs from patients with chronic obstructive pulmonary disease.* Histochem Cell Biol, 2001. 115(2): p. 117-24.

204. Tanaka, A., et al., *An angiotensin II receptor antagonist reduces myocardial damage in an animal model of myocarditis.* Circulation, 1994. 90(4): p. 2051-5.
205. Otsuka, M., et al., *Reduction of bleomycin induced lung fibrosis by candesartan cilexetil, an angiotensin II type 1 receptor antagonist.* Thorax, 2004. 59(1): p. 31-8.
206. Fishel, R.S., et al., *Fibroblast growth factor stimulates angiotensin converting enzyme expression in vascular smooth muscle cells. Possible mediator of the response to vascular injury.* J Clin Invest, 1995. 95(1): p. 377-87.
207. Schunkert, H., et al., *Alteration of growth responses in established cardiac pressure overload hypertrophy in rats with aortic banding.* J Clin Invest, 1995. 96(6): p. 2768-74.
208. McKay, S., et al., *Angiotensin II induces hypertrophy of human airway smooth muscle cells: expression of transcription factors and transforming growth factor-beta1.* Am J Respir Cell Mol Biol, 1998. 18(6): p. 823-33.
209. Sun, Y., et al., *Angiotensin II, transforming growth factor-beta1 and repair in the infarcted heart.* J Mol Cell Cardiol, 1998. 30(8): p. 1559-69.
210. Okada, K., et al., *Angiotensin-converting enzyme inhibition delays pulmonary vascular neointimal formation.* Am J Respir Crit Care Med, 1998. 158(3): p. 939-50.
211. Border, W.A. and N.A. Noble, *Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis.* Hypertension, 1998. 31(1 Pt 2): p. 181-8.
212. Ali, S.M., et al., *Angiotensin-converting enzyme inhibition attenuates proteinuria and renal TGF-beta 1 mRNA expression in rats with chronic renal disease.* Pharmacology, 1998. 57(1): p. 20-7.
213. Sun, Y. and K.T. Weber, *Cardiac remodelling by fibrous tissue: role of local factors and circulating hormones.* Ann Med, 1998. 30 Suppl 1: p. 3-8.
214. Ng, K.K. and J.R. Vane, *Conversion of angiotensin I to angiotensin II.* Nature, 1967. 216(5117): p. 762-6.
215. Ryan, J.W., et al., *Subcellular localization of pulmonary antiotensin-converting enzyme (kininase II).* Biochem J, 1975. 146(2): p. 497-9.
216. Yasuda, S., et al., *Angiotensin-converting enzyme inhibition restores hepatocyte growth factor production in patients with congestive heart failure.* Hypertension, 1999. 33(6): p. 1374-8.
217. Sacco, G., et al., *Cardioprotective effects of zofenopril, a new angiotensin-converting enzyme inhibitor, on doxorubicin-induced cardiotoxicity in the rat.* Eur J Pharmacol, 2001. 414(1): p. 71-8.
218. Campbell, S.E. and L.C. Katwa, *Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts.* J Mol Cell Cardiol, 1997. 29(7): p. 1947-58.
219. Song, L., et al., *Kinetic alterations of angiotensin-II and nitric oxide in radiation pulmonary fibrosis.* J Environ Pathol Toxicol Oncol, 1998. 17(2): p. 141-50.
220. Wenz, M., et al., *Inhaled nitric oxide does not change transpulmonary angiotensin II formation in patients with acute respiratory distress syndrome.* Chest, 1997. 112(2): p. 478-83.

221. Lu, L., M.T. Quinn, and Y. Sun, *Oxidative stress in the infarcted heart: role of de novo angiotensin II production.* Biochem Biophys Res Commun, 2004. 325(3): p. 943-51.
222. Ozawa, Y., et al., *The angiotensin II type 2 receptor primarily inhibits cell growth via pertussis toxin-sensitive G proteins.* Biochem Biophys Res Commun, 1996. 228(2): p. 328-33.
223. Wang, R., et al., *Angiotensin II induces apoptosis in human and rat alveolar epithelial cells.* Am J Physiol, 1999. 276(5 Pt 1): p. L885-9.
224. Wang, R., et al., *Apoptosis of lung epithelial cells in response to TNF-alpha requires angiotensin II generation de novo.* J Cell Physiol, 2000. 185(2): p. 253-9.
225. Admiraal, P.J., et al., *Metabolism and production of angiotensin I in different vascular beds in subjects with hypertension.* Hypertension, 1990. 15(1): p. 44-55.
226. Filippatos, G.S., et al., *Regulation of apoptosis by vasoactive peptides.* Am J Physiol Lung Cell Mol Physiol, 2001. 281(4): p. L749-61.
227. Li, D., et al., *Proapoptotic effects of ANG II in human coronary artery endothelial cells: role of AT1 receptor and PKC activation.* Am J Physiol, 1999. 276(3 Pt 2): p. H786-92.
228. Uhal, B.D., et al., *Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism.* Am J Physiol, 1998. 275(5 Pt 1): p. L1013-7.
229. Wang, R., et al., *Fas-induced apoptosis of alveolar epithelial cells requires ANG II generation and receptor interaction.* Am J Physiol, 1999. 277(6 Pt 1): p. L1245-50.
230. Horiuchi, M., M. Akishita, and V.J. Dzau, *Molecular and cellular mechanism of angiotensin II-mediated apoptosis.* Endocr Res, 1998. 24(3-4): p. 307-14.
231. Yamada, T., M. Horiuchi, and V.J. Dzau, *Angiotensin II type 2 receptor mediates programmed cell death.* Proc Natl Acad Sci U S A, 1996. 93(1): p. 156-60.
232. Papp, M., et al., *Angiotensin receptor subtype AT(1) mediates alveolar epithelial cell apoptosis in response to ANG II.* Am J Physiol Lung Cell Mol Physiol, 2002. 282(4): p. L713-8.
233. Horiuchi, M., et al., *Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis.* J Biol Chem, 1997. 272(30): p. 19022-6.
234. Rossig, L., et al., *Angiotensin II-induced upregulation of MAP kinase phosphatase-3 mRNA levels mediates endothelial cell apoptosis.* Basic Res Cardiol, 2002. 97(1): p. 1-8.
235. Feng, Y.H., Y. Sun, and J.G. Douglas, *Gbeta gamma -independent constitutive association of Galpha s with SHP-1 and angiotensin II receptor AT2 is essential in AT2-mediated ITIM-independent activation of SHP-1.* Proc Natl Acad Sci U S A, 2002. 99(19): p. 12049-54.
236. Hunyady, L., T. Balla, and K.J. Catt, *The ligand binding site of the angiotensin AT1 receptor.* Trends Pharmacol Sci, 1996. 17(4): p. 135-40.

237. Yamano, Y., et al., *Identification of amino acid residues of rat angiotensin II receptor for ligand binding by site directed mutagenesis.* Biochem Biophys Res Commun, 1992. 187(3): p. 1426-31.
238. Ohyama, K., et al., *Domains for G-protein coupling in angiotensin II receptor type I: studies by site-directed mutagenesis.* Biochem Biophys Res Commun, 1992. 189(2): p. 677-83.
239. Linseman, D.A., C.W. Benjamin, and D.A. Jones, *Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells.* J Biol Chem, 1995. 270(21): p. 12563-8.
240. Molloy, C.J., D.S. Taylor, and H. Weber, *Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells.* J Biol Chem, 1993. 268(10): p. 7338-45.
241. Giasson, E. and S. Meloche, *Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells.* J Biol Chem, 1995. 270(10): p. 5225-31.
242. Bhat, G.J., et al., *Angiotensin II stimulates sis-inducing factor-like DNA binding activity. Evidence that the AT1A receptor activates transcription factor-Stat91 and/or a related protein.* J Biol Chem, 1994. 269(50): p. 31443-9.
243. Marrero, M.B., et al., *Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor.* Nature, 1995. 375(6528): p. 247-50.
244. Nahmias, C. and A.D. Strosberg, *The angiotensin AT2 receptor: searching for signal-transduction pathways and physiological function.* Trends Pharmacol Sci, 1995. 16(7): p. 223-5.
245. Shanmugam, S., P. Corvol, and J.M. Gasc, *Angiotensin II type 2 receptor mRNA expression in the developing cardiopulmonary system of the rat.* Hypertension, 1996. 28(1): p. 91-7.
246. Lazard, D., et al., *Characterization of a membrane glycoprotein having pharmacological and biochemical properties of an AT2 angiotensin II receptor from human myometrium.* Eur J Biochem, 1994. 220(3): p. 919-26.
247. Ozono, R., et al., *Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney.* Hypertension, 1997. 30(5): p. 1238-46.
248. Touyz, R.M., et al., *Role of AT2 receptors in angiotensin II-stimulated contraction of small mesenteric arteries in young SHR.* Hypertension, 1999. 33(1 Pt 2): p. 366-72.
249. Touyz, R.M. and E.L. Schiffrin, *Angiotensin II regulates vascular smooth muscle cell pH, contraction, and growth via tyrosine kinase-dependent signaling pathways.* Hypertension, 1997. 30(2 Pt 1): p. 222-9.
250. Wang, Z.Q., et al., *Differential regulation of renal angiotensin subtype AT1A and AT2 receptor protein in rats with angiotensin-dependent hypertension.* Hypertension, 1999. 33(1): p. 96-101.
251. Bottari, S.P., et al., *Angiotensin II AT2 receptors do not interact with guanine nucleotide binding proteins.* Eur J Pharmacol, 1991. 207(2): p. 157-63.
252. Pucell, A.G., et al., *Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor.* Endocrinology, 1991. 128(4): p. 1947-59.
253. Stoll, M., et al., *The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells.* J Clin Invest, 1995. 95(2): p. 651-7.

254. Matsubara, H., *Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases.* Circ Res, 1998. 83(12): p. 1182-91.
255. Horiuchi, M., M. Akishita, and V.J. Dzau, *Recent progress in angiotensin II type 2 receptor research in the cardiovascular system.* Hypertension, 1999. 33(2): p. 613-21.
256. Lo, M., et al., *Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats.* J Clin Invest, 1995. 95(3): p. 1394-7.
257. Janiak, P., et al., *Role of angiotensin subtype 2 receptor in neointima formation after vascular injury.* Hypertension, 1992. 20(6): p. 737-45.
258. Nakajima, M., et al., *The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer.* Proc Natl Acad Sci U S A, 1995. 92(23): p. 10663-7.
259. Levy, B.I., et al., *Chronic blockade of AT2-subtype receptors prevents the effect of angiotensin II on the rat vascular structure.* J Clin Invest, 1996. 98(2): p. 418-25.
260. Laflamme, L., et al., *Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors.* J Biol Chem, 1996. 271(37): p. 22729-35.
261. Mifune, M., et al., *Angiotensin II type 2 receptors stimulate collagen synthesis in cultured vascular smooth muscle cells.* Hypertension, 2000. 36(5): p. 845-50.
262. Waseda, Y., et al., *Angiotensin II type 2 receptor antagonist reduces bleomycin-induced pulmonary fibrosis in mice.* Respir Res, 2008. 9: p. 43.
263. Nouet, S. and C. Nahmias, *Signal transduction from the angiotensin II AT2 receptor.* Trends Endocrinol Metab, 2000. 11(1): p. 1-6.
264. Bottari, S.P., et al., *Characterization and distribution of angiotensin II binding sites in fetal and neonatal astrocytes from different rat brain regions.* Brain Res, 1992. 585(1-2): p. 372-6.
265. Brechler, V., et al., *Angiotensin II stimulates protein tyrosine phosphatase activity through a G-protein independent mechanism.* Receptors Channels, 1994. 2(2): p. 89-98.
266. Bedecs, K., et al., *Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase.* Biochem J, 1997. 325 (Pt 2): p. 449-54.
267. Huang, X.C., C. Sumners, and E.M. Richards, *Angiotensin II stimulates protein phosphatase 2A activity in cultured neuronal cells via type 2 receptors in a pertussis toxin sensitive fashion.* Adv Exp Med Biol, 1996. 396: p. 209-15.
268. Yamada, H., et al., *AT2 receptor and vascular smooth muscle cell differentiation in vascular development.* Hypertension, 1999. 33(6): p. 1414-9.
269. Huang, X.C., E.M. Richards, and C. Sumners, *Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors.* J Biol Chem, 1996. 271(26): p. 15635-41.
270. Akishita, M., et al., *Expression of the AT2 receptor developmentally programs extracellular signal-regulated kinase activity and influences fetal vascular growth.* J Clin Invest, 1999. 103(1): p. 63-71.

271. Fischer, T.A., et al., *Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes.* Am J Physiol, 1998. 275(3 Pt 2): p. H906-16.
272. Heximer, S.P., et al., *Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice.* J Clin Invest, 2003. 111(8): p. 1259.
273. Cui, T., et al., *Pivotal role of tyrosine phosphatase SHP-1 in AT2 receptor-mediated apoptosis in rat fetal vascular smooth muscle cell.* Cardiovasc Res, 2001. 49(4): p. 863-71.
274. Hannan, R.E., et al., *Differential regulation by AT(1) and AT(2) receptors of angiotensin II-stimulated cyclic GMP production in rat uterine artery and aorta.* Br J Pharmacol, 2004. 141(6): p. 1024-31.
275. Saito, S., et al., *Angiotensin II activates endothelial constitutive nitric oxide synthase via AT1 receptors.* Hypertens Res, 1996. 19(3): p. 201-6.
276. Wiemer, G., et al., *The possible role of angiotensin II subtype AT2 receptors in endothelial cells and isolated ischemic rat hearts.* J Hypertens Suppl, 1993. 11(5): p. S234-5.
277. Seyedi, N., et al., *Coronary kinin generation mediates nitric oxide release after angiotensin receptor stimulation.* Hypertension, 1995. 26(1): p. 164-70.
278. Thorup, C., et al., *Angiotensin-II stimulates nitric oxide release in isolated perfused renal resistance arteries.* Pflugers Arch, 1998. 435(3): p. 432-4.
279. Siragy, H.M., *The potential role of the angiotensin subtype 2 receptor in cardiovascular protection.* Curr Hypertens Rep, 2009. 11(4): p. 260-2.
280. Haithcock, D., et al., *Renal proximal tubular AT2 receptor: signaling and transport.* J Am Soc Nephrol, 1999. 10 Suppl 11: p. S69-74.
281. Kohout, T.A. and T.B. Rogers, *Angiotensin II activates the Na+/HCO3- symport through a phosphoinositide-independent mechanism in cardiac cells.* J Biol Chem, 1995. 270(35): p. 20432-8.
282. Sandmann, S., et al., *Differential effects of angiotensin AT1 and AT2 receptors on the expression, translation and function of the Na+-H+ exchanger and Na+-HCO3- symporter in the rat heart after myocardial infarction.* J Am Coll Cardiol, 2001. 37(8): p. 2154-65.
283. Dulin, N.O., et al., *Phospholipase A2-mediated activation of mitogen-activated protein kinase by angiotensin II.* Proc Natl Acad Sci U S A, 1998. 95(14): p. 8098-102.
284. Jiao, H., et al., *Arachidonic acid mediates angiotensin II effects on p21ras in renal proximal tubular cells via the tyrosine kinase-Shc-Grb2-Sos pathway.* Proc Natl Acad Sci U S A, 1998. 95(13): p. 7417-21.
285. Stroth, U., et al., *Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: role of the AT2 receptor.* Brain Res Mol Brain Res, 1998. 53(1-2): p. 187-95.
286. Wruck, C.J., et al., *Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated Golgi protein.* Arterioscler Thromb Vasc Biol, 2005. 25(1): p. 57-64.
287. Miura, S. and S.S. Karnik, *Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis.* Embo J, 2000. 19(15): p. 4026-35.

288. Lehtonen, J.Y., et al., *Activation of the de novo biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis.* J Biol Chem, 1999. 274(24): p. 16901-6.
289. Gallinat, S., et al., *AT2 receptor stimulation induces generation of ceramides in PC12W cells.* FEBS Lett, 1999. 443(1): p. 75-9.
290. de Gasparo, M., et al., *International union of pharmacology. XXIII. The angiotensin II receptors.* Pharmacol Rev, 2000. 52(3): p. 415-72.
291. Kang, J., P. Posner, and C. Sumners, *Angiotensin II type 2 receptor stimulation of neuronal K+ currents involves an inhibitory GTP binding protein.* Am J Physiol, 1994. 267(5 Pt 1): p. C1389-97.
292. Zhang, J. and R.E. Pratt, *The AT2 receptor selectively associates with Gialpha2 and Gialpha3 in the rat fetus.* J Biol Chem, 1996. 271(25): p. 15026-33.
293. Hardie, D.G., D. Carling, and M. Carlson, *The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell?* Annu Rev Biochem, 1998. 67: p. 821-55.
294. Hattori, Y., et al., *Activation of AMP-activated protein kinase enhances angiotensin ii-induced proliferation in cardiac fibroblasts.* Hypertension, 2006. 47(2): p. 265-70.
295. Nagata, D., et al., *AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation.* Circulation, 2004. 110(4): p. 444-51.
296. Hattori, N., et al., *The plasminogen activation system reduces fibrosis in the lung by a hepatocyte growth factor-dependent mechanism.* Am J Pathol, 2004. 164(3): p. 1091-8.
297. Sankarshanan, M., et al., *Identification of a novel lipid raft-targeting motif in Src homology 2-containing phosphatase 1.* J Immunol, 2007. 179(1): p. 483-90.
298. Yi, T.L., J.L. Cleveland, and J.N. Ihle, *Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13.* Mol Cell Biol, 1992. 12(2): p. 836-46.
299. Valencia, A.M., et al., *Identification of a protein-tyrosine phosphatase (SHP1) different from that associated with acid phosphatase in rat prostate.* FEBS Lett, 1997. 406(1-2): p. 42-8.
300. Chong, Z.Z. and K. Maiese, *The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury.* Histol Histopathol, 2007. 22(11): p. 1251-67.
301. Yang, J., et al., *Crystal structure of the catalytic domain of protein-tyrosine phosphatase SHP-1.* J Biol Chem, 1998. 273(43): p. 28199-207.
302. Bennett, A.M., et al., *Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression.* Mol Cell Biol, 1996. 16(3): p. 1189-202.
303. Lorenz, U., et al., *Lck-dependent tyrosyl phosphorylation of the phosphotyrosine phosphatase SH-PTP1 in murine T cells.* Mol Cell Biol, 1994. 14(3): p. 1824-34.
304. Bennett, A.M., et al., *Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras.* Proc Natl Acad Sci U S A, 1994. 91(15): p. 7335-9.

305. Vogel, W. and A. Ullrich, *Multiple in vivo phosphorylated tyrosine phosphatase SHP-2 engages binding to Grb2 via tyrosine 584.* Cell Growth Differ, 1996. 7(12): p. 1589-97.
306. Feng, G.S., et al., *Receptor-binding, tyrosine phosphorylation and chromosome localization of the mouse SH2-containing phosphotyrosine phosphatase Syp.* Oncogene, 1994. 9(6): p. 1545-50.
307. Bialy, L. and H. Waldmann, *Inhibitors of protein tyrosine phosphatases: next-generation drugs?* Angew Chem Int Ed Engl, 2005. 44(25): p. 3814-39.
308. Yamauchi, K., et al., *Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling.* Proc Natl Acad Sci U S A, 1995. 92(3): p. 664-8.
309. You, M. and Z. Zhao, *Positive effects of SH2 domain-containing tyrosine phosphatase SHP-1 on epidermal growth factor- and interferon-gamma-stimulated activation of STAT transcription factors in HeLa cells.* J Biol Chem, 1997. 272(37): p. 23376-81.
310. Yi, T., et al., *Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis.* Mol Cell Biol, 1993. 13(12): p. 7577-86.
311. Pani, G., et al., *Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling.* J Exp Med, 1995. 181(6): p. 2077-84.
312. Kim, C.H., et al., *Abnormal chemokine-induced responses of immature and mature hematopoietic cells from motheaten mice implicate the protein tyrosine phosphatase SHP-1 in chemokine responses.* J Exp Med, 1999. 190(5): p. 681-90.
313. Chen, C.H., et al., *Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts.* Mol Pharmacol, 2006. 69(4): p. 1347-55.
314. Herrera Abreu, M.T., et al., *Tyrosine phosphatase SHP-2 regulates IL-1 signaling in fibroblasts through focal adhesions.* J Cell Physiol, 2006. 207(1): p. 132-43.
315. Chen, J., et al., *A negative role of SHP-2 tyrosine phosphatase in growth factor-dependent hematopoietic cell survival.* Oncogene, 2004. 23(20): p. 3659-69.
316. Shetty, S., M.R. Gyetko, and A.P. Mazar, *Induction of p53 by urokinase in lung epithelial cells.* J Biol Chem, 2005. 280(30): p. 28133-41.
317. Yu, L., et al., *JAK2 and SHP2 reciprocally regulate tyrosine phosphorylation and stability of proapoptotic protein ASK1.* J Biol Chem, 2009. 284(20): p. 13481-8.
318. Sue, Y.M., et al., *Urotensin II induces transactivation of the epidermal growth factor receptor via transient oxidation of SHP-2 in the rat renal tubular cell line NRK-52E.* Growth Factors, 2009. 27(3): p. 155-62.
319. Escande, M.L., N. Gas, and B.J. Stevens, *Immunolocalization of the 100 K nucleolar protein in CHO cells.* Biol Cell, 1985. 53(2): p. 99-109.
320. Smetana, K., et al., *Immunofluorescence studies on proteins B23 and C23 in nucleoli of human lymphocytes.* Exp Cell Res, 1984. 152(1): p. 195-203.

321. Jordan, G., *At the heart of the nucleolus.* Nature, 1987. 329(6139): p. 489-90.
322. Lapeyre, B., H. Bourbon, and F. Amalric, *Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence.* Proc Natl Acad Sci U S A, 1987. 84(6): p. 1472-6.
323. Deng, J.S., B. Ballou, and J.K. Hofmeister, *Internalization of anti-nucleolin antibody into viable HEp-2 cells.* Mol Biol Rep, 1996. 23(3-4): p. 191-5.
324. Kleinman, H.K., et al., *Identification of a 110-kDa nonintegrin cell surface laminin-binding protein which recognizes an A chain neurite-promoting peptide.* Arch Biochem Biophys, 1991. 290(2): p. 320-5.
325. Yu, D., M.Z. Schwartz, and R. Petryshyn, *Effect of laminin on the nuclear localization of nucleolin in rat intestinal epithelial IEC-6 cells.* Biochem Biophys Res Commun, 1998. 247(1): p. 186-92.
326. Zaidi, S.H. and J.S. Malter, *Nucleolin and heterogeneous nuclear ribonucleoprotein C proteins specifically interact with the 3'-untranslated region of amyloid protein precursor mRNA.* J Biol Chem, 1995. 270(29): p. 17292-8.
327. Ishikawa, F., et al., *Nuclear proteins that bind the pre-mRNA 3' splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)n.* Mol Cell Biol, 1993. 13(7): p. 4301-10.
328. Nigg, E.A., *Nucleocytoplasmic transport: signals, mechanisms and regulation.* Nature, 1997. 386(6627): p. 779-87.
329. Schwab, M.S. and C. Dreyer, *Protein phosphorylation sites regulate the function of the bipartite NLS of nucleolin.* Eur J Cell Biol, 1997. 73(4): p. 287-97.
330. Haneji, T., *[Protein phosphatases and nucleolin in osteoblastic cells: cleavage of nucleolin in apoptotic cells].* Kaibogaku Zasshi, 2003. 78(3): p. 71-5.
331. Wang, H.Y., X.L. Liu, and B.M. Jiang, *[Changes of nucleolin expression and cellular localization during HUVEC apoptosis induced by hydrogen peroxide].* Zhong Nan Da Xue Xue Bao Yi Xue Ban, 2008. 33(6): p. 488-93.
332. Wang, K.K., et al., *[Effect of nucleolin down-regulation on the proliferation and apoptosis in C2C12 cells].* Zhong Nan Da Xue Xue Bao Yi Xue Ban, 2005. 30(2): p. 125-9.
333. Erard, M.S., et al., *A major nucleolar protein, nucleolin, induces chromatin decondensation by binding to histone H1.* Eur J Biochem, 1988. 175(3): p. 525-30.
334. Herrera, A.H. and M.O. Olson, *Association of protein C23 with rapidly labeled nucleolar RNA.* Biochemistry, 1986. 25(20): p. 6258-64.
335. Bugler, B., et al., *RNA binding fragments from nucleolin contain the ribonucleoprotein consensus sequence.* J Biol Chem, 1987. 262(23): p. 10922-5.
336. Belenguer, P., et al., *Protein kinase NII and the regulation of rDNA transcription in mammalian cells.* Nucleic Acids Res, 1989. 17(16): p. 6625-36.
337. Yang, T.H., et al., *Purification and characterization of nucleolin and its identification as a transcription repressor.* Mol Cell Biol, 1994. 14(9): p. 6068-74.

338. Bouche, G., et al., *Interrelations between the maturation of a 100 kDa nucleolar protein and pre rRNA synthesis in CHO cells.* Nucleic Acids Res, 1984. 12(7): p. 3025-35.
339. Olson, M.O., et al., *Interaction of nucleolar phosphoprotein C23 with cloned segments of rat ribosomal deoxyribonucleic acid.* Biochemistry, 1983. 22(14): p. 3345-51.
340. Egyhazi, E., et al., *Heat-shock-specific phosphorylation and transcriptional activity of RNA polymerase II.* Exp Cell Res, 1998. 242(1): p. 211-21.
341. Ginisty, H., F. Amalric, and P. Bouvet, *Nucleolin functions in the first step of ribosomal RNA processing.* Embo J, 1998. 17(5): p. 1476-86.
342. Bouvet, P., et al., *Nucleolin interacts with several ribosomal proteins through its RGG domain.* J Biol Chem, 1998. 273(30): p. 19025-9.
343. Derenzini, M., et al., *The quantity of nucleolar proteins nucleolin and protein B23 is related to cell doubling time in human cancer cells.* Lab Invest, 1995. 73(4): p. 497-502.
344. Brockstedt, E., et al., *Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. Cleavage of heterogeneous nuclear ribonucleoprotein A1 by caspase 3.* J Biol Chem, 1998. 273(43): p. 28057-64.
345. Borer, R.A., et al., *Major nucleolar proteins shuttle between nucleus and cytoplasm.* Cell, 1989. 56(3): p. 379-90.
346. Yurkova, M.S. and M.T. Murray, *A translation regulatory particle containing the Xenopus oocyte Y box protein mRNP3+4.* J Biol Chem, 1997. 272(16): p. 10870-6.
347. Zaidi, S.H., R. Denman, and J.S. Malter, *Multiple proteins interact at a unique cis-element in the 3'-untranslated region of amyloid precursor protein mRNA.* J Biol Chem, 1994. 269(39): p. 24000-6.
348. Tuteja, R. and N. Tuteja, *Nucleolin: a multifunctional major nucleolar phosphoprotein.* Crit Rev Biochem Mol Biol, 1998. 33(6): p. 407-36.
349. Westmark, C.J. and J.S. Malter, *Up-regulation of nucleolin mRNA and protein in peripheral blood mononuclear cells by extracellular-regulated kinase.* J Biol Chem, 2001. 276(2): p. 1119-26.
350. Erard, M., F. Lakhdar-Ghazal, and F. Amalric, *Repeat peptide motifs which contain beta-turns and modulate DNA condensation in chromatin.* Eur J Biochem, 1990. 191(1): p. 19-26.
351. Srivastava, M., et al., *Cloning and sequencing of the human nucleolin cDNA.* FEBS Lett, 1989. 250(1): p. 99-105.
352. Goyette, M., et al., *Regulated transcription of c-Ki-ras and c-myc during compensatory growth of rat liver.* Mol Cell Biol, 1984. 4(8): p. 1493-8.
353. Cavaille, J., M. Nicoloso, and J.P. Bachellerie, *Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides.* Nature, 1996. 383(6602): p. 732-5.
354. Caizergues-Ferrer, M., et al., *Nucleolin from Xenopus laevis: cDNA cloning and expression during development.* Genes Dev, 1989. 3(3): p. 324-33.
355. Belenguer, P., et al., *Mitosis-specific phosphorylation of nucleolin by p34cdc2 protein kinase.* Mol Cell Biol, 1990. 10(7): p. 3607-18.

356. Peter, M., et al., *Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase.* Cell, 1990. 60(5): p. 791-801.
357. Creancier, L., et al., *Determination of the functional domains involved in nucleolar targeting of nucleolin.* Mol Biol Cell, 1993. 4(12): p. 1239-50.
358. Sipos, K. and M.O. Olson, *Nucleolin promotes secondary structure in ribosomal RNA.* Biochem Biophys Res Commun, 1991. 177(2): p. 673-8.
359. Heine, M.A., M.L. Rankin, and P.J. DiMario, *The Gly/Arg-rich (GAR) domain of Xenopus nucleolin facilitates in vitro nucleic acid binding and in vivo nucleolar localization.* Mol Biol Cell, 1993. 4(11): p. 1189-204.
360. Ghisolfi, L., et al., *The glycine-rich domain of nucleolin has an unusual supersecondary structure responsible for its RNA-helix-destabilizing properties.* J Biol Chem, 1992. 267(5): p. 2955-9.
361. Najbauer, J., et al., *Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins.* J Biol Chem, 1993. 268(14): p. 10501-9.
362. Schmidt-Zachmann, M.S., et al., *Nuclear export of proteins: the role of nuclear retention.* Cell, 1993. 74(3): p. 493-504.
363. Bandziulis, R.J., M.S. Swanson, and G. Dreyfuss, *RNA-binding proteins as developmental regulators.* Genes Dev, 1989. 3(4): p. 431-7.
364. Query, C.C., R.C. Bentley, and J.D. Keene, *A common RNA recognition motif identified within a defined U1 RNA binding domain of the 70K U1 snRNP protein.* Cell, 1989. 57(1): p. 89-101.
365. Ghisolfi-Nieto, L., et al., *Nucleolin is a sequence-specific RNA-binding protein: characterization of targets on pre-ribosomal RNA.* J Mol Biol, 1996. 260(1): p. 34-53.
366. Bouvet, P., et al., *RNA recognition by the joint action of two nucleolin RNA-binding domains: genetic analysis and structural modeling.* Embo J, 1997. 16(17): p. 5235-46.
367. Allain, F.H., et al., *Solution structure of the two N-terminal RNA-binding domains of nucleolin and NMR study of the interaction with its RNA target.* J Mol Biol, 2000. 303(2): p. 227-41.
368. Allain, F.H., et al., *Molecular basis of sequence-specific recognition of pre-ribosomal RNA by nucleolin.* Embo J, 2000. 19(24): p. 6870-81.
369. Shim, J. and M. Karin, *The control of mRNA stability in response to extracellular stimuli.* Mol Cells, 2002. 14(3): p. 323-31.
370. Chen, C.Y. and A.B. Shyu, *Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements.* Mol Cell Biol, 1994. 14(12): p. 8471-82.
371. Meyer, S., C. Temme, and E. Wahle, *Messenger RNA turnover in eukaryotes: pathways and enzymes.* Crit Rev Biochem Mol Biol, 2004. 39(4): p. 197-216.
372. Mukherjee, D., et al., *The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements.* Embo J, 2002. 21(1-2): p. 165-74.
373. Grzybowska, E.A., A. Wilczynska, and J.A. Siedlecki, *Regulatory functions of 3'UTRs.* Biochem Biophys Res Commun, 2001. 288(2): p. 291-5.

374. Audic, Y. and R.S. Hartley, *Post-transcriptional regulation in cancer.* Biol Cell, 2004. 96(7): p. 479-98.
375. Barreau, C., L. Paillard, and H.B. Osborne, *AU-rich elements and associated factors: are there unifying principles?* Nucleic Acids Res, 2005. 33(22): p. 7138-50.
376. Pesole, G., et al., *Structural and compositional features of untranslated regions of eukaryotic mRNAs.* Gene, 1997. 205(1-2): p. 95-102.
377. Dreyfuss, G., V.N. Kim, and N. Kataoka, *Messenger-RNA-binding proteins and the messages they carry.* Nat Rev Mol Cell Biol, 2002. 3(3): p. 195-205.
378. Lasko, P., *Gene regulation at the RNA layer: RNA binding proteins in intercellular signaling networks.* Sci STKE, 2003. 2003(179): p. RE6.
379. Kuersten, S. and E.B. Goodwin, *The power of the 3' UTR: translational control and development.* Nat Rev Genet, 2003. 4(8): p. 626-37.
380. Alberta, J.A., K. Rundell, and C.D. Stiles, *Identification of an activity that interacts with the 3'-untranslated region of c-myc mRNA and the role of its target sequence in mediating rapid mRNA degradation.* J Biol Chem, 1994. 269(6): p. 4532-8.
381. Chen, C.Y. and A.B. Shyu, *AU-rich elements: characterization and importance in mRNA degradation.* Trends Biochem Sci, 1995. 20(11): p. 465-70.
382. Ross, J., *mRNA stability in mammalian cells.* Microbiol Rev, 1995. 59(3): p. 423-50.
383. Keene, J.D., *Why is Hu where? Shuttling of early-response-gene messenger RNA subsets.* Proc Natl Acad Sci U S A, 1999. 96(1): p. 5-7.
384. Brennan, C.M. and J.A. Steitz, *HuR and mRNA stability.* Cell Mol Life Sci, 2001. 58(2): p. 266-77.
385. Bobola, N., et al., *Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells.* Cell, 1996. 84(5): p. 699-709.
386. Oleynikov, Y. and R.H. Singer, *Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization.* Curr Biol, 2003. 13(3): p. 199-207.
387. Ross, A.F., et al., *Characterization of a beta-actin mRNA zipcode-binding protein.* Mol Cell Biol, 1997. 17(4): p. 2158-65.
388. Ayala, Y.M., et al., *Human, Drosophila, and C.elegans TDP43: nucleic acid binding properties and splicing regulatory function.* J Mol Biol, 2005. 348(3): p. 575-88.
389. Chou, C.F., et al., *Tethering KSRP, a decay-promoting AU-rich element-binding protein, to mRNAs elicits mRNA decay.* Mol Cell Biol, 2006. 26(10): p. 3695-706.
390. Pan, Y.X., H. Chen, and M.S. Kilberg, *Interaction of RNA-binding proteins HuR and AUF1 with the human ATF3 mRNA 3'-untranslated region regulates its amino acid limitation-induced stabilization.* J Biol Chem, 2005. 280(41): p. 34609-16.
391. Sengupta, T.K., et al., *Identification of nucleolin as an AU-rich element binding protein involved in bcl-2 mRNA stabilization.* J Biol Chem, 2004. 279(12): p. 10855-63.

392. Rajagopalan, L.E., et al., *hnRNP C increases amyloid precursor protein (APP) production by stabilizing APP mRNA.* Nucleic Acids Res, 1998. 26(14): p. 3418-23.
393. Hanakahi, L.A., et al., *Nucleolin is one component of the B cell-specific transcription factor and switch region binding protein, LR1.* Proc Natl Acad Sci U S A, 1997. 94(8): p. 3605-10.
394. Chen, C.Y., et al., *Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation.* Genes Dev, 2000. 14(10): p. 1236-48.
395. Jiang, Y., X.S. Xu, and J.E. Russell, *A nucleolin-binding 3' untranslated region element stabilizes beta-globin mRNA in vivo.* Mol Cell Biol, 2006. 26(6): p. 2419-29.
396. Stoker, M., et al., *Scatter factor is a fibroblast-derived modulator of epithelial cell mobility.* Nature, 1987. 327(6119): p. 239-42.
397. Stoker, M. and M. Perryman, *An epithelial scatter factor released by embryo fibroblasts.* J Cell Sci, 1985. 77: p. 209-23.
398. Nakamura, T., et al., *Molecular cloning and expression of human hepatocyte growth factor.* Nature, 1989. 342(6248): p. 440-3.
399. Nakamura, T., *Structure and function of hepatocyte growth factor.* Prog Growth Factor Res, 1991. 3(1): p. 67-85.
400. Jiang, W.G., M.B. Hallett, and M.C. Puntis, *Hepatocyte growth factor/scatter factor, liver regeneration and cancer metastasis.* Br J Surg, 1993. 80(11): p. 1368-73.
401. Jiang, W.G. and S. Hiscox, *Hepatocyte growth factor/scatter factor, a cytokine playing multiple and converse roles.* Histol Histopathol, 1997. 12(2): p. 537-55.
402. Matsumoto, K. and T. Nakamura, *Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration.* Biochem Biophys Res Commun, 1997. 239(3): p. 639-44.
403. Rosen, E.M., S.K. Nigam, and I.D. Goldberg, *Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction.* J Cell Biol, 1994. 127(6 Pt 2): p. 1783-7.
404. Naldini, L., et al., *Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor.* Embo J, 1991. 10(10): p. 2867-78.
405. Dunsmore, S.E., et al., *Mechanisms of hepatocyte growth factor stimulation of keratinocyte metalloproteinase production.* J Biol Chem, 1996. 271(40): p. 24576-82.
406. Jiang, W., et al., *Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer.* Crit Rev Oncol Hematol, 1999. 29(3): p. 209-48.
407. Miyazawa, K., et al., *Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII.* J Biol Chem, 1993. 268(14): p. 10024-8.

408. Miyazawa, K., T. Shimomura, and N. Kitamura, *Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator.* J Biol Chem, 1996. 271(7): p. 3615-8.
409. Shimomura, T., et al., *Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator.* Eur J Biochem, 1995. 229(1): p. 257-61.
410. Shimomura, T., et al., *Activation of the zymogen of hepatocyte growth factor activator by thrombin.* J Biol Chem, 1993. 268(30): p. 22927-32.
411. Naka, D., et al., *Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer.* J Biol Chem, 1992. 267(28): p. 20114-9.
412. Weidner, K.M., M. Sachs, and W. Birchmeier, *The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells.* J Cell Biol, 1993. 121(1): p. 145-54.
413. Kawaida, K., et al., *Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice.* Proc Natl Acad Sci U S A, 1994. 91(10): p. 4357-61.
414. Schmassmann, A., et al., *Roles of hepatocyte growth factor and its receptor Met during gastric ulcer healing in rats.* Gastroenterology, 1997. 113(6): p. 1858-72.
415. Ohmichi, H., K. Matsumoto, and T. Nakamura, *In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration.* Am J Physiol, 1996. 270(6 Pt 1): p. L1031-9.
416. Burr, A.W., et al., *Anti-hepatocyte growth factor antibody inhibits hepatocyte proliferation during liver regeneration.* J Pathol, 1998. 185(3): p. 298-302.
417. Yasuda, S., et al., *Enhanced secretion of cardiac hepatocyte growth factor from an infarct region is associated with less severe ventricular enlargement and improved cardiac function.* J Am Coll Cardiol, 2000. 36(1): p. 115-21.
418. Miura, Y., et al., *Hepatocyte growth factor stimulates proliferation and migration during wound healing of retinal pigment epithelial cells in vitro.* Jpn J Ophthalmol, 2003. 47(3): p. 268-75.
419. Watanabe, M., et al., *Hepatocyte growth factor gene transfer to alveolar septa for effective suppression of lung fibrosis.* Mol Ther, 2005. 12(1): p. 58-67.
420. Umeda, Y., et al., *Skeletal muscle targeting in vivo electroporation-mediated HGF gene therapy of bleomycin-induced pulmonary fibrosis in mice.* Lab Invest, 2004. 84(7): p. 836-44.
421. Dohi, M., et al., *Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis.* Am J Respir Crit Care Med, 2000. 162(6): p. 2302-7.
422. Yaekashiwa, M., et al., *Simultaneous or delayed administration of hepatocyte growth factor equally represses the fibrotic changes in murine lung injury induced by bleomycin. A morphologic study.* Am J Respir Crit Care Med, 1997. 156(6): p. 1937-44.
423. Cramer, T., et al., *Hepatocyte growth factor and c-Met expression in rat and human liver fibrosis.* Liver Int, 2004. 24(4): p. 335-44.

424. Tahara, M., et al., *Hepatocyte growth factor leads to recovery from alcohol-induced fatty liver in rats.* J Clin Invest, 1999. 103(3): p. 313-20.
425. Komamura, K., et al., *Treatment of dilated cardiomyopathy with electroporation of hepatocyte growth factor gene into skeletal muscle.* Hypertension, 2004. 44(3): p. 365-71.
426. Matsumori, A., et al., *Increased circulating hepatocyte growth factor in the early stage of acute myocardial infarction.* Biochem Biophys Res Commun, 1996. 221(2): p. 391-5.
427. Dai, C. and Y. Liu, *Hepatocyte growth factor antagonizes the profibrotic action of TGF-beta1 in mesangial cells by stabilizing Smad transcriptional corepressor TGIF.* J Am Soc Nephrol, 2004. 15(6): p. 1402-12.
428. Mizuno, S., et al., *Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease.* J Clin Invest, 1998. 101(9): p. 1827-34.
429. Ware, L.B. and M.A. Matthay, *Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair.* Am J Physiol Lung Cell Mol Physiol, 2002. 282(5): p. L924-40.
430. Panos, R.J., R. Patel, and P.M. Bak, *Intratracheal administration of hepatocyte growth factor/scatter factor stimulates rat alveolar type II cell proliferation in vivo.* Am J Respir Cell Mol Biol, 1996. 15(5): p. 574-81.
431. Liu, X.L., et al., *The protective effect of hepatocyte growth-promoting factor (pHGF) against hydrogen peroxide-induced acute lung injury in rats.* Med Electron Microsc, 2001. 34(2): p. 92-102.
432. Mars, W.M., R. Zarnegar, and G.K. Michalopoulos, *Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA.* Am J Pathol, 1993. 143(3): p. 949-58.
433. Cooper, C.S., *The met oncogene: from detection by transfection to transmembrane receptor for hepatocyte growth factor.* Oncogene, 1992. 7(1): p. 3-7.
434. Bottaro, D.P., et al., *Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product.* Science, 1991. 251(4995): p. 802-4.
435. Dean, M., et al., *The human met oncogene is related to the tyrosine kinase oncogenes.* Nature, 1985. 318(6044): p. 385-8.
436. Giordano, S., et al., *Tyrosine kinase receptor indistinguishable from the c-met protein.* Nature, 1989. 339(6220): p. 155-6.
437. Giordano, S., et al., *Biosynthesis of the protein encoded by the c-met proto-oncogene.* Oncogene, 1989. 4(11): p. 1383-8.
438. Lee, C.C. and K.M. Yamada, *Alternatively spliced juxtamembrane domain of a tyrosine kinase receptor is a multifunctional regulatory site. Deletion alters cellular tyrosine phosphorylation pattern and facilitates binding of phosphatidylinositol-3-OH kinase to the hepatocyte growth factor receptor.* J Biol Chem, 1995. 270(2): p. 507-10.
439. Park, M., et al., *Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors.* Proc Natl Acad Sci U S A, 1987. 84(18): p. 6379-83.

440. Giordano, S., et al., *Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene.* Proc Natl Acad Sci U S A, 1993. 90(2): p. 649-53.
441. Rubin, J.S., D.P. Bottaro, and S.A. Aaronson, *Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product.* Biochim Biophys Acta, 1993. 1155(3): p. 357-71.
442. Epstein, J.A., et al., *Pax3 modulates expression of the c-Met receptor during limb muscle development.* Proc Natl Acad Sci U S A, 1996. 93(9): p. 4213-8.
443. Honda, S., et al., *Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor.* Brain Res Mol Brain Res, 1995. 32(2): p. 197-210.
444. Di Renzo, M.F., et al., *Selective expression of the Met/HGF receptor in human central nervous system microglia.* Oncogene, 1993. 8(1): p. 219-22.
445. Yamada, T., et al., *Immunohistochemistry with antibodies to hepatocyte growth factor and its receptor protein (c-MET) in human brain tissues.* Brain Res, 1994. 637(1-2): p. 308-12.
446. Bussolino, F., et al., *Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth.* J Cell Biol, 1992. 119(3): p. 629-41.
447. Halaban, R., J.S. Rubin, and W. White, *met and HGF-SF in normal melanocytes and melanoma cells.* Exs, 1993. 65: p. 329-39.
448. Chan, A.M., et al., *Identification of a competitive HGF antagonist encoded by an alternative transcript.* Science, 1991. 254(5036): p. 1382-5.
449. Hartmann, G., et al., *A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis.* Proc Natl Acad Sci U S A, 1992. 89(23): p. 11574-8.
450. Lokker, N.A., et al., *Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding.* Embo J, 1992. 11(7): p. 2503-10.
451. Lokker, N.A. and P.J. Godowski, *Generation and characterization of a competitive antagonist of human hepatocyte growth factor, HGF/NK1.* J Biol Chem, 1993. 268(23): p. 17145-50.
452. Lokker, N.A., L.G. Presta, and P.J. Godowski, *Mutational analysis and molecular modeling of the N-terminal kringle-containing domain of hepatocyte growth factor identifies amino acid side chains important for interaction with the c-Met receptor.* Protein Eng, 1994. 7(7): p. 895-903.
453. Matsumoto, K., et al., *Deletion of kringle domains or the N-terminal hairpin structure in hepatocyte growth factor results in marked decreases in related biological activities.* Biochem Biophys Res Commun, 1991. 181(2): p. 691-9.
454. Okigaki, M., et al., *Functional characterization of human hepatocyte growth factor mutants obtained by deletion of structural domains.* Biochemistry, 1992. 31(40): p. 9555-61.
455. Faletto, D.L., et al., *Signal transduction in c-met mediated motogenesis.* Exs, 1993. 65: p. 107-30.

456. Ponzetto, C., et al., *A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family.* Cell, 1994. 77(2): p. 261-71.
457. Songyang, Z., et al., *SH2 domains recognize specific phosphopeptide sequences.* Cell, 1993. 72(5): p. 767-78.
458. Songyang, Z., et al., *A single point mutation switches the specificity of group III Src homology (SH) 2 domains to that of group I SH2 domains.* J Biol Chem, 1995. 270(44): p. 26029-32.
459. Boccaccio, C., et al., *Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF.* J Biol Chem, 1994. 269(17): p. 12846-51.
460. Moghul, A., et al., *Modulation of c-MET proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-MET transcript.* Oncogene, 1994. 9(7): p. 2045-52.
461. Chen, P., et al., *Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement.* J Cell Biol, 1994. 127(3): p. 847-57.
462. Zhang, Y.W. and G.F. Vande Woude, *HGF/SF-met signaling in the control of branching morphogenesis and invasion.* J Cell Biochem, 2003. 88(2): p. 408-17.
463. Corso, S., P.M. Comoglio, and S. Giordano, *Cancer therapy: can the challenge be MET?* Trends Mol Med, 2005. 11(6): p. 284-92.
464. Beviglia, L. and R.H. Kramer, *HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.* Int J Cancer, 1999. 83(5): p. 640-9.
465. Chen, H.C., et al., *Tyrosine phosphorylation of focal adhesion kinase stimulated by hepatocyte growth factor leads to mitogen-activated protein kinase activation.* J Biol Chem, 1998. 273(40): p. 25777-82.
466. Liu, Z.X., et al., *Hepatocyte growth factor induces ERK-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association.* J Biol Chem, 2002. 277(12): p. 10452-8.
467. Birukova, A.A., et al., *Paxillin is involved in the differential regulation of endothelial barrier by HGF and VEGF.* Am J Respir Cell Mol Biol, 2009. 40(1): p. 99-107.
468. Shelly, C. and R. Herrera, *Activation of SGK1 by HGF, Rac1 and integrin-mediated cell adhesion in MDCK cells: PI-3K-dependent and -independent pathways.* J Cell Sci, 2002. 115(Pt 9): p. 1985-93.
469. Trusolino, L., A. Bertotti, and P.M. Comoglio, *A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth.* Cell, 2001. 107(5): p. 643-54.
470. Schaper, F., et al., *Hepatocyte growth factor/scatter factor (HGF/SF) signals via the STAT3/APRF transcription factor in human hepatoma cells and hepatocytes.* FEBS Lett, 1997. 405(1): p. 99-103.
471. Lee, B.S., et al., *Hepatocyte growth factor induces delayed STAT3 phosphorylation through interleukin-6 expression.* Cell Signal, 2009. 21(3): p. 419-27.

472. Berchtold, S., et al., *Dominant negative variants of the SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 (janus kinase 2) and induction of Stat5 (signal transducer and activator of transcription 5)-dependent transcription.* Mol Endocrinol, 1998. 12(4): p. 556-67.
473. Noguchi, T., et al., *Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation.* Mol Cell Biol, 1994. 14(10): p. 6674-82.
474. Takagi, M., et al., *Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin.* Cell, 2005. 123(1): p. 49-63.
475. McClenahan, D., et al., *Effects of lipopolysaccharide and Mannheimia haemolytica leukotoxin on bovine lung microvascular endothelial cells and alveolar epithelial cells.* Clin Vaccine Immunol, 2008. 15(2): p. 338-47.
476. Kitta, K., et al., *Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis.* Free Radic Biol Med, 2001. 31(7): p. 902-10.
477. Krown, K.A., et al., *Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes: involvement of the sphingolipid signaling cascade in cardiac cell death.* J Clin Invest, 1996. 98: p. 2854-2865.
478. Mondello, C. and A.I. Scovassi, *Apoptosis: a way to maintain healthy individuals.* Subcell Biochem. 50: p. 307-23.
479. Yang, E., et al., *Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death.* Cell, 1995. 80(2): p. 285-91.
480. Sedlak, T.W., et al., *Multiple Bcl-2 family members demonstrate selective dimerizations with Bax.* Proc Natl Acad Sci U S A, 1995. 92(17): p. 7834-8.
481. Oltvai, Z.N., C.L. Milliman, and S.J. Korsmeyer, *Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death.* Cell, 1993. 74(4): p. 609-19.
482. Boise, L.H., et al., *Bcl-2 and Bcl-2-related proteins in apoptosis regulation.* Curr Top Microbiol Immunol, 1995. 200: p. 107-21.
483. Yang, E. and S.J. Korsmeyer, *Molecular thanatopsis: a discourse on the BCL2 family and cell death.* Blood, 1996. 88(2): p. 386-401.
484. Reed, J.C., *Bcl-2: prevention of apoptosis as a mechanism of drug resistance.* Hematol Oncol Clin North Am, 1995. 9(2): p. 451-73.
485. Wei, M.C., et al., *Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death.* Science, 2001. 292(5517): p. 727-30.
486. Lindsten, T., et al., *The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues.* Mol Cell, 2000. 6(6): p. 1389-99.
487. Yin, X.M., Z.N. Oltvai, and S.J. Korsmeyer, *BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax.* Nature, 1994. 369(6478): p. 321-3.
488. Selvakumaran, M., et al., *Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways.* Oncogene, 1994. 9(6): p. 1791-8.
489. Miyashita, T., et al., *Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo.* Oncogene, 1994. 9(6): p. 1799-805.

490. Matsubara, H., et al., *Effect of angiotensin II type 2 receptor on tyrosine kinase Pyk2 and c-Jun NH2-terminal kinase via SHP-1 tyrosine phosphatase activity: evidence from vascular-targeted transgenic mice of AT2 receptor.* Biochem Biophys Res Commun, 2001. 282(5): p. 1085-91.
491. Wu, L., et al., *Regulation of inhibitory protein-kappaB and monocyte chemoattractant protein-1 by angiotensin II type 2 receptor-activated Src homology protein tyrosine phosphatase-1 in fetal vascular smooth muscle cells.* Mol Endocrinol, 2004. 18(3): p. 666-78.
492. Li, J.M., et al., *Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1.* Mol Endocrinol, 2007. 21(2): p. 499-511.
493. Alvarez, S.E., et al., *Involvement of c-Src tyrosine kinase in SHP-1 phosphatase activation by Ang II AT2 receptors in rat fetal tissues.* J Cell Biochem, 2008. 105(3): p. 703-11.
494. Marshall, R.P., et al., *Angiotensin II and the fibroproliferative response to acute lung injury.* Am J Physiol Lung Cell Mol Physiol, 2004. 286(1): p. L156-64.
495. Miura, S. and S.S. Karnik, *Angiotensin II type 1 and type 2 receptors bind angiotensin II through different types of epitope recognition.* J Hypertens, 1999. 17(3): p. 397-404.
496. Vogel, W., et al., *Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation.* Science, 1993. 259(5101): p. 1611-4.
497. Pazdrak, K., T. Adachi, and R. Alam, *Src homology 2 protein tyrosine phosphatase (SHPTP2)/Src homology 2 phosphatase 2 (SHP2) tyrosine phosphatase is a positive regulator of the interleukin 5 receptor signal transduction pathways leading to the prolongation of eosinophil survival.* J Exp Med, 1997. 186(4): p. 561-8.
498. Li, J., et al., *Angiotensin II type 2 receptor-dependent increases in nitric oxide synthase expression in the pulmonary endothelium is mediated via a G alpha i3/Ras/Raf/MAPK pathway.* Am J Physiol Cell Physiol, 2007. 292(6): p. C2185-96.
499. Pan, Z., et al., *The function study on the interaction between Grb2 and AMPK.* Mol Cell Biochem, 2008. 307(1-2): p. 121-7.
500. Tzatsos, A. and P.N. Tsichlis, *Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794.* J Biol Chem, 2007. 282(25): p. 18069-82.
501. Kim, Y.M., et al., *Involvement of AMPK signaling cascade in capsaicin-induced apoptosis of HT-29 colon cancer cells.* Ann N Y Acad Sci, 2007. 1095: p. 496-503.
502. Hwang, J.T., et al., *Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway.* Cancer Lett, 2007. 247(1): p. 115-21.
503. Li, P., et al., *Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.* Cell, 1997. 91(4): p. 479-89.
504. Liu, X., et al., *Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c.* Cell, 1996. 86(1): p. 147-57.

505. Schlegel, J., et al., *CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis.* J Biol Chem, 1996. 271(4): p. 1841-4.
506. Wang, X., et al., *Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis.* Embo J, 1996. 15(5): p. 1012-20.
507. Wang, W., et al., *AMP-activated kinase regulates cytoplasmic HuR.* Mol Cell Biol, 2002. 22(10): p. 3425-36.
508. Chen, F.W. and Y.A. Ioannou, *Ribosomal proteins in cell proliferation and apoptosis.* Int Rev Immunol, 1999. 18(5-6): p. 429-48.
509. Buttgereit, F. and M.D. Brand, *A hierarchy of ATP-consuming processes in mammalian cells.* Biochem J, 1995. 312 (Pt 1): p. 163-7.
510. Horman, S., et al., *Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis.* Curr Biol, 2002. 12(16): p. 1419-23.
511. Browne, G.J., S.G. Finn, and C.G. Proud, *Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398.* J Biol Chem, 2004. 279(13): p. 12220-31.
512. Kimura, N., et al., *A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway.* Genes Cells, 2003. 8(1): p. 65-79.
513. Bolster, D.R., et al., *AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling.* J Biol Chem, 2002. 277(27): p. 23977-80.
514. Krause, U., L. Bertrand, and L. Hue, *Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes.* Eur J Biochem, 2002. 269(15): p. 3751-9.
515. Heitman, J., N.R. Movva, and M.N. Hall, *Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast.* Science, 1991. 253(5022): p. 905-9.
516. Kunz, J., et al., *Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression.* Cell, 1993. 73(3): p. 585-96.
517. Cardenas, M.E., et al., *The TOR signaling cascade regulates gene expression in response to nutrients.* Genes Dev, 1999. 13(24): p. 3271-9.
518. Hardwick, J.S., et al., *Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins.* Proc Natl Acad Sci U S A, 1999. 96(26): p. 14866-70.
519. Cardenas, M.E. and J. Heitman, *FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity.* Embo J, 1995. 14(23): p. 5892-907.
520. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. 115(5): p. 577-90.
521. Gao, X., et al., *Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling.* Nat Cell Biol, 2002. 4(9): p. 699-704.
522. Ban, J., et al., *Identification of a human cDNA encoding a novel Bcl-x isoform.* Biochem Biophys Res Commun, 1998. 248(1): p. 147-52.

523. Boise, L.H., et al., *bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death.* Cell, 1993. 74(4): p. 597-608.
524. Grillot, D.A., et al., *Genomic organization, promoter region analysis, and chromosome localization of the mouse bcl-x gene.* J Immunol, 1997. 158(10): p. 4750-7.
525. MacCarthy-Morrogh, L., et al., *Identification of a novel human BCL-X promoter and exon.* Oncogene, 2000. 19(48): p. 5534-8.
526. Cao, X., et al., *Up-regulation of Bcl-xl by hepatocyte growth factor in human mesothelioma cells involves ETS transcription factors.* Am J Pathol, 2009. 175(5): p. 2207-16.
527. Chen, C., L.C. Edelstein, and C. Gelinas, *The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L).* Mol Cell Biol, 2000. 20(8): p. 2687-95.
528. Sevilla, L., et al., *The Ets2 transcription factor inhibits apoptosis induced by colony-stimulating factor 1 deprivation of macrophages through a Bcl-xL-dependent mechanism.* Mol Cell Biol, 1999. 19(4): p. 2624-34.
529. Kirito, K., et al., *Thrombopoietin regulates Bcl-xL gene expression through Stat5 and phosphatidylinositol 3-kinase activation pathways.* J Biol Chem, 2002. 277(10): p. 8329-37.
530. Tokumitsu, H., et al., *Regulatory mechanism of Ca2+/calmodulin-dependent protein kinase kinase.* J Biol Chem, 2000. 275(26): p. 20090-5.
531. Lasa, M., et al., *Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade.* Mol Cell Biol, 2000. 20(12): p. 4265-74.
532. Cok, S.J. and A.R. Morrison, *The 3'-untranslated region of murine cyclooxygenase-2 contains multiple regulatory elements that alter message stability and translational efficiency.* J Biol Chem, 2001. 276(25): p. 23179-85.
533. Treisman, R., *Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences.* Cell, 1985. 42(3): p. 889-902.
534. Peppel, K., J.M. Vinci, and C. Baglioni, *The AU-rich sequences in the 3' untranslated region mediate the increased turnover of interferon mRNA induced by glucocorticoids.* J Exp Med, 1991. 173(2): p. 349-55.
535. Dixon, D.A., et al., *Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells.* J Clin Invest, 2001. 108(11): p. 1657-65.
536. Shaw, G. and R. Kamen, *A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation.* Cell, 1986. 46(5): p. 659-67.
537. Stoecklin, G., S. Hahn, and C. Moroni, *Functional hierarchy of AUUUA motifs in mediating rapid interleukin-3 mRNA decay.* J Biol Chem, 1994. 269(46): p. 28591-7.
538. Jones, T.R. and M.D. Cole, *Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences.* Mol Cell Biol, 1987. 7(12): p. 4513-21.

539. Zhang, Y., et al., *Nucleolin links to arsenic-induced stabilization of GADD45alpha mRNA.* Nucleic Acids Res, 2006. 34(2): p. 485-95.
540. Glisovic, T., et al., *RNA-binding proteins and post-transcriptional gene regulation.* FEBS Lett, 2008. 582(14): p. 1977-86.
541. Bailey-Serres, J., R. Sorenson, and P. Juntawong, *Getting the message across: cytoplasmic ribonucleoprotein complexes.* Trends Plant Sci, 2009. 14(8): p. 443-53.
542. Yang, C., D.A. Maiguel, and F. Carrier, *Identification of nucleolin and nucleophosmin as genotoxic stress-responsive RNA-binding proteins.* Nucleic Acids Res, 2002. 30(10): p. 2251-60.
543. Srivastava, M. and H.B. Pollard, *Molecular dissectionof nucleolin's role in growth and cell proliferation: new insights.* FASEB J, 1999. 13: p. 1911-1922.
544. Otake, Y., et al., *Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA.* Blood, 2007. 109(7): p. 3069-75.
545. Angelov, D., et al., *Nucleolin is a histone chaperone with FACT-like activity and assists remodeling of nucleosomes.* Embo J, 2006. 25(8): p. 1669-79.
546. Bouche, G., et al., *Activation of rDNA transcription by FGF-2: key role of protein kinase CKII.* Cell Mol Biol Res, 1994. 40(5-6): p. 547-54.
547. Ugrinova, I., et al., *Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication.* BMC Mol Biol, 2007. 8: p. 66.
548. Kito, S., et al., *Cleavage of nucleolin and argyrophilic nucleolar organizer region associated proteins in apoptosis-induced cells.* Biochem Biophys Res Commun, 2003. 300(4): p. 950-6.
549. Datta, S.R., A. Brunet, and M.E. Greenberg, *Cellular survival: a play in three Akts.* Genes Dev, 1999. 13(22): p. 2905-27.
550. Barel, M., et al., *Activation of Epstein-Barr virus/C3d receptor (gp140, CR2, CD21) on human cell surface triggers pp60src and Akt-GSK3 activities upstream and downstream to PI 3-kinase, respectively.* Eur J Immunol, 2003. 33(9): p. 2557-66.
551. Borgatti, P., et al., *Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associates with the nuclear matrix protein nucleolin.* J Cell Physiol, 2003. 196(1): p. 79-88.
552. Ricupero, D.A., et al., *Phosphatidylinositol 3-kinase-dependent stabilization of alpha1(I) collagen mRNA in human lung fibroblasts.* Am J Physiol Cell Physiol, 2001. 281(1): p. C99-C105.
553. Tabet, F., et al., *Redox-sensitive signaling by angiotensin II involves oxidative inactivation and blunted phosphorylation of protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells from SHR.* Circ Res, 2008. 103(2): p. 149-58.
554. Dohi, M., et al., *Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis.* Am J Respir Crit Care Med, 2000. 162(6): p. 2302-7.
555. Kawaida, K., et al., *Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice.* Proc Natl Acad Sci U S A, 1994. 91(10): p. 4357-61.

556. Kitta, K., et al., *Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis.* Free Radic Biol Med, 2001. 31(7): p. 902-10.
557. Kosai, K., et al., *Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice.* Hepatology, 1999. 30(1): p. 151-9.
558. Fan, S., et al., *Scatter factor protects epithelial and carcinoma cells against apoptosis induced by DNA-damaging agents.* Oncogene, 1998. 17(2): p. 131-41.
559. Kosai, K., et al., *Abrogation of Fas-induced fulminant hepatic failure in mice by hepatocyte growth factor.* Biochem Biophys Res Commun, 1998. 244(3): p. 683-90.
560. Liu, Y., A.M. Sun, and L.D. Dworkin, *Hepatocyte growth factor protects renal epithelial cells from apoptotic cell death.* Biochem Biophys Res Commun, 1998. 246(3): p. 821-6.
561. Zhang, J., J. Yang, and Y. Liu, *Role of Bcl-xL Induction in HGF-mediated Renal Epithelial Cell Survival after Oxidant Stress.* Int J Clin Exp Pathol, 2008. 1(3): p. 242-53.
562. Nishino, M., et al., *Hepatocyte growth factor improves survival after partial hepatectomy in cirrhotic rats suppressing apoptosis of hepatocytes.* Surgery, 2008. 144(3): p. 374-84.
563. Adams, J.M. and S. Cory, *The Bcl-2 protein family: arbiters of cell survival.* Science, 1998. 281(5381): p. 1322-6.
564. Chinnaiyan, A.M., et al., *Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death.* Science, 1997. 275(5303): p. 1122-6.
565. Day, R.M., et al., *Differential signaling by alternative HGF isoforms through c-Met: activation of both MAP kinase and PI 3-kinase pathways is insufficient for mitogenesis.* Oncogene, 1999. 18(22): p. 3399-406.
566. Fan, S., et al., *The cytokine hepatocyte growth factor/scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3' kinase.* Oncogene, 2000. 19(18): p. 2212-23.
567. Takebayashi, T., et al., *Hepatocyte growth factor/scatter factor modulates cell motility, proliferation, and proteoglycan synthesis of chondrocytes.* J Cell Biol, 1995. 129(5): p. 1411-9.
568. Nakanishi, K., et al., *Hepatocyte growth factor promotes migration of human hepatocellular carcinoma via phosphatidylinositol 3-kinase.* Clin Exp Metastasis, 1999. 17(6): p. 507-14.
569. Royal, I., T.M. Fournier, and M. Park, *Differential requirement of Grb2 and PI3-kinase in HGF/SF-induced cell motility and tubulogenesis.* J Cell Physiol, 1997. 173(2): p. 196-201.
570. Royal, I. and M. Park, *Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase.* J Biol Chem, 1995. 270(46): p. 27780-7.
571. Potempa, S. and A.J. Ridley, *Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly.* Mol Biol Cell, 1998. 9(8): p. 2185-200.

572. Bardelli, A., et al., *Concomitant activation of pathways downstream of Grb2 and PI 3-kinase is required for MET-mediated metastasis.* Oncogene, 1999. 18(5): p. 1139-46.
573. del Peso, L., et al., *Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt.* Science, 1997. 278(5338): p. 687-9.
574. Hsu, S.Y., et al., *Interference of BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis in mammalian cells by 14-3-3 isoforms and P11.* Mol Endocrinol, 1997. 11(12): p. 1858-67.
575. Cardone, M.H., et al., *Regulation of cell death protease caspase-9 by phosphorylation.* Science, 1998. 282(5392): p. 1318-21.
576. Matsumoto, K. and T. Nakamura, *Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases.* Kidney Int, 2001. 59(6): p. 2023-38.
577. Rosario, M. and W. Birchmeier, *How to make tubes: signaling by the Met receptor tyrosine kinase.* Trends Cell Biol, 2003. 13(6): p. 328-35.
578. Funakoshi, H. and T. Nakamura, *Hepatocyte growth factor: from diagnosis to clinical applications.* Clin Chim Acta, 2003. 327(1-2): p. 1-23.
579. Seger, R. and E.G. Krebs, *The MAPK signaling cascade.* Faseb J, 1995. 9(9): p. 726-35.
580. Sergeant, N., et al., *Stimulation of DNA synthesis and cell proliferation of human mammary myoepithelial-like cells by hepatocyte growth factor/scatter factor depends on heparan sulfate proteoglycans and sustained phosphorylation of mitogen-activated protein kinases p42/44.* J Biol Chem, 2000. 275(22): p. 17094-9.
581. Chen, R.H., C. Sarnecki, and J. Blenis, *Nuclear localization and regulation of erk- and rsk-encoded protein kinases.* Mol Cell Biol, 1992. 12(3): p. 915-27.
582. Stolz, D.B. and G.K. Michalopoulos, *Comparative effects of hepatocyte growth factor and epidermal growth factor on motility, morphology, mitogenesis, and signal transduction of primary rat hepatocytes.* J Cell Biochem, 1994. 55(4): p. 445-64.
583. Adachi, T., et al., *Mitogen-activated protein kinase activation in hepatocyte growth factor-stimulated rat hepatocytes: involvement of protein tyrosine kinase and protein kinase C.* Hepatology, 1996. 23(5): p. 1244-53.
584. Herrera, R., *Modulation of hepatocyte growth factor-induced scattering of HT29 colon carcinoma cells. Involvement of the MAPK pathway.* J Cell Sci, 1998. 111 (Pt 8): p. 1039-49.
585. Lai, J.F., et al., *Involvement of focal adhesion kinase in hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells.* J Biol Chem, 2000. 275(11): p. 7474-80.
586. Nakagami, H., et al., *Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells.* Hypertension, 2001. 37(2 Part 2): p. 581-6.
587. Gao, M., et al., *Hepatocyte growth factor/scatter factor blocks the mitochondrial pathway of apoptosis signaling in breast cancer cells.* J Biol Chem, 2001. 276(50): p. 47257-65.

588. Xiao, G.H., et al., *Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways.* Proc Natl Acad Sci U S A, 2001. 98(1): p. 247-52.
589. Ito, T., et al., *Bcl-2 phosphorylation required for anti-apoptosis function.* J Biol Chem, 1997. 272(18): p. 11671-3.
590. Wang, X., et al., *A mechanism of cell survival: sequestration of Fas by the HGF receptor Met.* Mol Cell, 2002. 9(2): p. 411-21.
591. Liu, Y., *Hepatocyte growth factor promotes renal epithelial cell survival by dual mechanisms.* Am J Physiol, 1999. 277(4 Pt 2): p. F624-33.
592. Veiga, E. and P. Cossart, *Listeria InlB takes a different route to met.* Cell, 2007. 130(2): p. 218-9.
593. Bierne, H. and P. Cossart, *InlB, a surface protein of Listeria monocytogenes that behaves as an invasin and a growth factor.* J Cell Sci, 2002. 115(Pt 17): p. 3357-67.
594. Bierne, H., et al., *The invasion protein InIB from Listeria monocytogenes activates PLC-gamma1 downstream from PI 3-kinase.* Cell Microbiol, 2000. 2(6): p. 465-76.
595. Bosse, T., et al., *Cdc42 and phosphoinositide 3-kinase drive Rac-mediated actin polymerization downstream of c-Met in distinct and common pathways.* Mol Cell Biol, 2007. 27(19): p. 6615-28.
596. Ireton, K., et al., *A role for phosphoinositide 3-kinase in bacterial invasion.* Science, 1996. 274(5288): p. 780-2.